Sexual Selection in House Mice (*Mus musculus domesticus*) and the Role of a Selfish Genetic Element

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Summary

According to the Mendelian rules of inheritance, every chromosome or allele of a diploid organism has a 50% chance of being transmitted to a given offspring. These rules are violated by selfish genetic elements which distort transmission to increase their own representation in the next generation. This contrasts with the traditional Darwinian view of evolution, under which genetic variants that confer a fitness benefit to their bearers should propagate. Indeed, selfish genetic elements can increase in frequency even if they decrease the fitness of the whole organism, which causes intra-genomic conflict between the selfish genetic element and the rest of the genome. The genome as a whole will thus attempt to suppress the costly actions of selfish genetic elements. This thesis explores how the behaviour of the organism might act as a suppressor of intra-genomic conflict in house mice.

The *t* haplotype is a well-known example of a selfish genetic element that is found in house mice. It is a large stretch of DNA that manipulates male gametogenesis to subvert Mendelian inheritance (i.e., it shows 'meiotic drive'). Moreover, the *t* haplotype carries recessive embryonic lethal mutations that impose a strong fitness cost on the rest of the genome. Its selfishness at the gamete level allows it to persist despite its negative effect of the fitness of its bearers. Given that females invest heavily into offspring, and that the *t* haplotype has severe negative effects on offspring viability, females should avoid fertilisation by males carrying the *t* haplotype (+/*t* males). Because the male drive mechanism affects sperm, postcopulatory sexual selection offers a particularly promising mechanism for avoiding fertilisation by +/*t* males. The first three chapters of this thesis investigate the effects of pre- and postcopulatory sexual selection on the *t* haplotype, and how the *t* haplotype may in turn affect sexual selection in house mice. Chapters 4 and 5 extend the investigation of male reproductive strategies in sperm competition.

Chapter 1 addresses how the *t* haplotype's manipulation of spermatogenesis affects the sperm competitiveness of +/*t* males. When a female mates with multiple males during a single reproductive episode, the sperm from different males can overlap and compete over fertilisation of the female's ova. The relative number and speed of a male's sperm determine his fertilisation likelihood. Because the *t* haplotype sabotages half of a +/*t* male's sperm, this should reduce the competitiveness of a +/*t* ejaculate against a wild type male's ejaculate. Moreover, the intra-ejaculate sabotage might come at an additional cost to the *t* bearing sperm. Females may benefit from this if mating with multiple males reduces the success of +/*t* males, which increases offspring viability and quality. The outcome of controlled sperm competition trials between +/*t* and +/+ males showed that +/*t* males are indeed severely disadvantaged against +/+ males, and that the strength of the effect is stronger than expected if the drive mechanism left *t* bearing sperm unaffected. As a consequence, multiply mated +/*t* females have more viable embryos than females mated only to a +/*t* male. Polyandry thus offers a mechanism for avoiding costly fertilisation by costly +/*t* males.

Chapter 2 expands the investigation of the effects of the +/*t* haplotype on sperm competition to investigate how reduced sperm competitiveness may have repercussions on male reproductive strategies. When wild type males mate with a previously unmated female, they sire the majority of her offspring, regardless of whether the female successively mates with an additional male. A

+/*t* male on the other hand loses more if a female he has mated with remates with another male, given the low sperm competitiveness of +/*t* males. We may thus expect +/*t* males to invest more into mate guarding to secure paternity. Interestingly, sperm competition between two +/*t* males showed that +/*t* males are generally disadvantaged in the first-to-mate role, which is in strong contrast with the general paternity advantage of wild type (+/+) males when first-to-mate. This accentuates the difference in the incentive for mate guarding between +/*t* and +/+ males. However, none of the hypothesised differences in reproductive strategies were found between +/*t* and +/+ males, suggesting that mate guarding might not be a feasible strategy for male house mice, or that the *t* haplotype is unable to exert control over polygenic behavioural traits.

In Chapter 3, active female discrimination against +/*t* males is investigated at different stages before and after mating. Previous research showed that females preferred the smell of +/+ males over that of +/*t* males, but a recent experiment that looked at an actual mating context found no evidence for precopulatory female discrimination against +/*t* males. The results reported in this thesis showed that females were not more likely to mate with +/+ males than with +/*t* males. Moreover, females did not accept ejaculation by +/+ males more quickly, were not more likely to remate after mating with a +/*t* male and did not show any evidence for preferential fertilisation by wild type sperm over *t* bearing sperm from within a +/*t* male's ejaculate. These findings indicate that females may not be able to detect male *t* genotype and to physiologically counteract male drive at the gamete level. Alternatively, females may simply rely on polyandry as an effective strategy against drive-mediated fitness costs.

Sperm competition favours male adaptations that increase competitive fertilisation success or decrease female multiple mating. Males of many species produce copulatory plugs that obstruct the female's genitals after ejaculation, which has generally been interpreted as a male adaptation to sperm competition. However, copulatory plugs might have other functions, and empirical tests of a function of the copulatory plug in sperm competition have provided mixed results over a variety of animal taxa. The results of Chapter 4 showed that male mice are strongly limited in the ejaculate components that the copulatory plug is made of, as evidenced by a reduction in plug size concomitant with repeated ejaculation. Males that produce smaller plugs appear to delay the ejaculation of rival males less, and possibly as a consequence, father fewer offspring. In Chapter 5, copulatory plugs were experimentally removed, and the consequences for copulatory behaviour and paternity were analysed in more detail. The results confirmed that large copulatory plugs delay rival male ejaculation, with positive consequences for the paternity success of first-to-mate males.

The research presented in this thesis demonstrates the strong impact of sexual selection—particularly of postcopulatory sexual selection—on the *t* haplotype, and highlights potential consequences for wild house mouse populations. Sexual behaviour at the organismal level that exposes the downside of the actions of male drive offers a way to suppress conflict at the gene level. This thesis also advances our understanding of mechanistic aspects of sperm competition and of the relationship between sperm features and fertilisation in a vertebrate model species.

ZUSAMMENFASSUNG

Gemäß den Mendel'schen Regeln der Vererbung hat jedes Chromosom oder Allel dieselbe 50% Chance an einen Nachkommen vererbt zu werden. Egoistische genetische Elemente brechen diese fairen Regeln und erhöhen damit ihre Repräsentation in der nachfolgenden Generation. Dies steht in Kontrast zur traditionell darwinistischen Sichtweise, gemäß welcher diejenigen genetischen Varianten prosperieren, welche ihrem Träger einen Fitness-Vorteil verschaffen. Tatsächlich können egoistische genetische Elemente sich selbst dann verbreiten, wenn sie einen negativen Effekt auf die Fitness ihres Trägers ausüben. Dies führt zu intra-genomischem Konflikt zwischen dem egoistischen genetischen Element und dem Rest des Genoms. Das Genom als Gesamtheit wird sich dagegen zur Wehr setzen, indem es versucht die schädlichen Aktionen von egoistischen genetischen Elementen zu unterbinden. Diese Dissertation beschäftigt sich damit, inwiefern das Verhalten des gesamten Organismus dazu dienen kann, intra-genomischen Konflikt zu unterbinden.

Der *t* Haplotyp, der bei Hausmäusen vorkommt, ist ein bekanntes Beispiel eines egoistischen genetischen Elements. Es ist ein langer Chromosomenabschnitt welcher die Spermiogenese dahingehend manipuliert, um die Mendel'schen Vererbungsregeln zu umgehen. Darüber hinaus sind auf dem *t* Haplotyp rezessive Mutationen zu finden, die zum frühen Tod von Embryonen führen, welche für den *t* Haplotyp homozygot sind (d.h. zwei Kopien besitzen). Dies bedeutet Fitness-Kosten für den Rest des Genoms, da durch diesen lethalen Effekt die Anzahl Nachkommen verringert wird. Trotz seines homozygot negativen Effekts kann der *t* Haplotyp bestehen, weil er sich durch seinen Betrug bei der Vererbung einen genügend großen Vorteil verschafft. Der negative Effekt des *t* Haplotyps auf das Überleben der Embryonen führt zu der Erwartung, dass Weibchen die Befruchtung durch ein den *t* Haplotyp tragendes Männchen (+/*t* Männchen) vermeiden sollten, zumal Weibchen viel Energie in die Schwangerschaft investieren. Post-kopulatorische sexuelle Selektion scheint besonders vielversprechend um Befruchtung durch +/*t* Männchen zu verhindern, da der Betrugsmechanismus des *t* Haplotyps auf der Manipulation der Spermien beruht. Die ersten drei Kapitel dieser Dissertation befassen sich mit dem Einfluss prä- und post-kopulatorischer sexueller Selektion auf den *t* Haplotyp; und damit, wie im Gegenzug der *t* Haplotyp Prozesse der sexuellen Selektion bei Hausmäusen beeinflussen mag. Die beiden abschließenden Kapitel untersuchen Strategien von männlichen Hausmäusen im Bezug auf Spermienkonkurrenz.

KAPITEL 1 untersucht, wie sich die Manipulation der Spermiogenese durch den *t* Haplotyp auf die Konkurrenzfähigkeit des Ejakulats von +/*t* Männchen auswirkt. Wenn sich ein Weibchen innerhalb eines einzigen Fruchtbarkeitszyklus mit mehreren Männchen verpaart, so können die vielen Spermien verschiedener Männchen über die Befruchtung einiger weniger Eizellen konkurrieren (Spermienkonkurrenz). Die Anzahl und Geschwindigkeit der Spermien sind hierbei entscheidend für den Befruchtungserfolg. Weil der *t* Haplotyp die Hälfte der Spermien eines +/*t* Männchens sabotiert (nämlich die + Spermien), könnte sich das negativ auf die Konkurrenzfähigkeit des +/*t* Ejakulates auswirken. Ausserdem ist es denkbar, dass der Sabotage-Akt auch negative Auswirkungen auf die *t* Spermien hat. Weibchen könnten von der Mehrfachverpaarung profitieren, wenn diese den Befruchtungserfolg der +/*t* Männchen

beeinträchtigt. Dies würde zu einer verbesserten Überlebenswahrscheinlichkeit der Embryonen führen. Um dies zu untersuchen wurden Weibchen in Experimenten im Labor mit jeweils einem $+/t$ und einem Wildtyp-Männchen (+/+) verpaart. Die Ergebnisse zeigten, dass $+/t$ Männchen in der Tat stark benachteiligt sind in der Spermienkonkurrenz, und dass der Effekt grösser ist, als wenn der Sabotage-Akt für die *t* Spermien schadlos überstanden würde. Die Auswirkungen waren für mehrfach verpaarte Weibchen positiv, da ihre Embryos eine erhöhte Überlebensrate hatten. Die Mehrfachverpaarung bietet demnach Weibchen eine Möglichkeit, die für ihre Nachkommen schädliche Befruchtung durch $+/t$ Männchen zu verhindern.

Kapitel 2 erweitert die Untersuchung der Spermienkonkurrenzfähigkeit von $+/t$ Männchen auf die Konsequenzen für die Paarungsstrategie von Männchen. Wenn Wildtyp-Männchen ein zuvor unverpaartes Weibchen begatten, dann stehen die Vaterschaftschancen gut, unabhängig davon ob anschließend noch ein zweites Männchen dasselbe Weibchen begattet. Für $+/t$ Männchen steht hingegen viel auf dem Spiel, wenn das Weibchen sich nach erfolgter Begattung noch weiter verpaart. Man könnte daher erwarten, dass $+/t$ Männchen vermehrt in die Bewachung von Weibchen investieren, um Spermienkonkurrenz zu verhindern. Interessanterweise schneiden $+/t$ Männchen in der Rolle des ersten Sexualpartners eines Weibchens schlecht ab, selbst wenn der Konkurrent ebenfalls ein $+/t$ Männchen ist. Dies verstärkt den Unterschied zwischen bezüglich der Vorteile von Bewachung zwischen $+/t$ und +/+ Männchen. Entgegen dieser Erwartung wurden keine Hinweise darauf gefunden, dass $+/t$ und +/+ Männchen unterschiedliche Fortpflanzungsstrategien verfolgen. Dies mag bedeuten, dass Hausmaus-Männchen Weibchen nicht effizient bewachen können. Als Alternative kommt in Frage, dass der *t* Haplotyp keine Kontrolle über das Verhalten der Männchen ausüben kann, zumal es sich um ein Merkmal handelt, welches wahrscheinlich durch eine große Anzahl Gene gesteuert wird.

In Kapitel 3 wird untersucht, inwiefern Weibchen auch vor und während der Kopulation gegen $+/t$ Männchen diskriminieren. Vorangehende Studien hatten gezeigt, dass Weibchen den Geruch von +/+ Männchen dem Geruch von $+/t$ Männchen vorzogen. Allerdings fand eine kürzlich veröffentlichte Studie keinen Hinweis auf eine solche Präferenz in einer Situation mit gleichzeitigem und unmittelbarem Zugang zu zwei Männchen. Die im Rahmen dieser Dissertation durchgeführten Experimente zeigten keinen Hinweis auf Diskriminierung gegen $+/t$ Männchen, weder was die Bereitschaft zur Paarung noch was das Verhalten während der Kopulation betrifft. Mit $+/t$ Männchen verpaarte Weibchen zeigten auch keine vermehrte Bereitwilligkeit zur Verpaarung mit einem zusätzlichen Männchen, und zeigten keine Hinweise darauf, dass für die Befruchtung aktiv + Spermien bevorzugt wurden. Dies könnte einerseits bedeuten, dass Weibchen nicht feststellen können welche Männchen den *t* Haplotyp tragen. Andererseits könnten sich Weibchen schlicht mit mehreren Männchen paaren und sich auf die Benachteiligung der $+/t$ Männchen durch die Spermienkonkurrenz verlassen.

Die Mehrfachverpaarung von Weibchen und die daraus resultierende Spermienkonkurrenz führen bei Männchen zu Anpassungen, die ihre Spermienkonkurrenzfähigkeit erhöhen und/oder das Risiko von Spermienkonkurrenz verringern. Männchen vieler Arten hinterlassen nach der Ejakulation Vaginalpfröpfe welche die weiblichen Genitalien ausfüllen. Dies wird allgemein als Anpassung an die Spermienkonkurrenz interpretiert, obwohl viele andere Funktionen ebenfalls denkbar sind und Experimente bei verschiedenen Arten gemischte Resultate hervorgebracht haben. Kapitel 4 zeigt, dass Hausmaus-Männchen stark limitiert sind, was die den Pfropf produzierenden Komponenten des Ejakulats betrifft. Dies wird dadurch ersichtlich, dass Männchen einer vorherigen Begattung folgend einen kleineren zweiten Pfropf hinterlassen.

Kleinere Vaginalpfröpfe gingen mit einer früheren Ejakulation eines nachfolgenden Rivalen und einem geringeren Vaterschaftserfolg einher. Im in KAPITEL 5 beschriebenen Experiment wurden Vaginalpfröpfe nach der Begattung entfernt, um den Zusammenhang zwischen Pfropf und Spermienkonkurrenz detaillierter untersuchen zu können. Die Ergebnisse bestätigten, dass der Vaginalpfropf die Begattung des Weibchens durch einen nachfolgenden Rivalen verzögert, was sich positiv auf den Vaterschaftserfolg des den Pfropf produzierenden Männchens niederschlägt.

Die in dieser Dissertation vorgelegten Ergebnisse belegen den Einfluss den die sexuelle Selektion—insbesondere die Spermienkonkurrenz—auf den Erfolg des *t* Haplotyps haben kann. Dies hat mögliche Konsequenzen für die Selektion in natürlichen Hausmaus-Populationen. Durch die Steuerung des Paarungsverhaltens des Organismus kann sich das Genom als Gesamtheit die Kehrseite des Sabotage-Aktes von egoistischen genetischen Elementen zu Nutze machen, um intra-genomischen Konflikt zu lösen. Diese Dissertation erweitert außerdem unser Verständnis mechanistischer Aspekte der Spermienkonkurrenz und des Befruchtungsvorgangs beim Modell-Organismus Maus.

MANUSCRIPTS SUBMITTED FOR PUBLICATION

I. **Sutter, A**, Lindholm, AK, **2015**. Detrimental effects of an autosomal selfish genetic element on sperm competitiveness in house mice.
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BMC Evolutionary Biology, **16**: 133.

III. **Sutter, A**, Lindholm, AK, **2016**. No evidence for female discrimination against genetically incompatible male house mice carrying a selfish genetic element.
Current Zoology, advance online access. DOI: 10.1093/cz/zow063

IV. [1] **Sutter, A**, Simmons, LW, Lindholm, AK & Firman, RC, **2016**. Function of copulatory plugs in house mice: mating behavior and paternity outcomes of rival males.
Behavioral Ecology, **27**: 185–195.

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General Introduction

Complex multicellular organisms rely on a high degree of cooperation between specialised cells and between the genes that constitute their genome. The genes must cooperate to form organisms in a way that makes them competitive against organisms composed by other gene collectives. However, this cooperation cannot be taken for granted (Burt & Trivers, 2006). The potential for conflict between alternative alleles at a given genetic locus is easily demonstrated in sexually reproducing diploid organisms. Only half of the alleles will be transmitted to any given offspring, as the sexual partner contributes the other half of the alleles of a diploid organism. According to the classic view of evolution by natural selection (Darwin, 1859), we expect alleles to strive if the covey a fitness benefit to their bearer. However, that is only one route that an allele can take to increase its future representation. An alternative way to strive is to simply cheat the transmission system to enhance one's chance of being part of the half of the alleles inherited by the offspring. If an allele is an effective cheater, it can even harm the organism that it resides in, as long as the benefit of cheating is greater than its negative effect on the whole organism (Bruck, 1957; Lyttle, 1993). Obviously, the alternative allele has opposing interests, because it will pay the direct cost of its opponent's selfish behaviour, i.e., it will be transmitted to fewer offspring. Additionally, if the selfish allele has negative effects on the organism as a whole, any allele that is not co-inherited with the selfish allele will pay the cost without gaining any benefit. This leads to conflict between two levels of selection, selection between the two alternative alleles, and selection between individuals with and without a selfish allele (Keller, 1999). Thus, the rest of the genome has a strong incentive to suppress the action of selfish alleles that impose costs to the genomic collective. A possible way to resolve the conflict is to make meiosis fair, ensuring that both alleles at a given locus have the exact same chance of transmission (Eshel, 1985; Haig & Grafen, 1991). If that is achieved, the interests of all alleles are aligned, because an individual allele has no way of telling whether or not it will be passed on at any specific reproductive event (Leigh, 1977; Okasha, 2012; Queller & Strassmann, 2013). This may account for the fact that meiosis usually works—it may have to, in order to maintain cooperation among the collective to maximise its competitiveness (Crow, 1991). However, this does not mean that conflict does not persist. In fact, the alleles remain in constant conflict over maximising selfish versus collective benefits. This thesis is concerned with a case study of a selfish genetic element that distorts fair Mendelian inheritance, and with how the rest of the genome might resolve intra-genomic conflict through altering the behaviour of the entire organism.

Genomic conflict caused by meiotic drive

Given how fundamental the conflict between individual alleles and the genomic collective is, it is perhaps of little surprise that there is a large diversity of cheating alleles. Selfish genetic elements are stretches of DNA that advance their own replication at the expense of the organism (they 'drive'; Burt & Trivers, 2006). Burt and Trivers (2006) outline three broad classes of how drive is achieved in eukaryotes: overreplication, gonotaxis and interference. Overreplication is the *modus operandi* of transposable elements, a well-known and particularly abundant class of selfish

genetic elements. Gonotaxis describes the preferential movement into the germ line, for example during female meiosis, where only one of the four products of meiotic divisions forms the functional ovum, whereas the other three become non-functional polar bodies. This example of cheating during meiosis itself has been termed meiotic drive (*sensu stricto*; Sandler & Novitski, 1957). Meiotic drive can however be defined more broadly to include not only manipulation of the meiotic process but generally of the premeiotic, meiotic or postmeiotic events (*sensu lato*; Lindholm et al., 2016). Gamete interference can be considered a form of meiotic drive, where the selfish allele kills or sabotages the gametes that do not carry a copy of that allele. In contrast to true meiotic drive, where one locus can be sufficient to manipulate meiosis in its favour, post-meiotic drive through interference is usually accomplished through the action of at least two loci, a distorter and a responder (Burt & Trivers, 2006). In the two molecularly best-understood drive systems, *Segregation Distorter* (SD) in *Drosophila melanogaster* and the *t* haplotype in house mice, the drive-specific distorter alleles attack a sensitive allele at the responder locus, whereas the driver has a non-sensitive allele at that locus (for reviews see Herrmann & Bauer, 2012 and Larracuente & Presgraves, 2012). Recombination between the distorter and the drive-specific responder allele will create suicidal combinations, so selection favours tight genetic linkage between the two (Charlesworth & Hartl, 1978; but see van Boven & Weissing, 2000). The reduced recombination rate on sex chromosomes is one of the reasons why drive is more likely to occur on sex chromosomes than on autosomes (Hurst & Pomiankowski, 1991; Lyttle, 1991; Jaenike, 2001). A drastic reduction of recombination can further be accomplished through chromosomal inversions, and drive systems show complex chromosomal rearrangements (Hammer et al., 1989; Dyer et al., 2007; Larracuente & Presgraves, 2012). The downside of a lack of recombination is the accumulation of recessive deleterious mutations by a process known as Muller's ratchet (Muller, 1964). Over time, this leads to negative fitness effects of drive systems, such as homozygote lethality of SD (Larracuente & Presgraves, 2012) and of the *t* haplotype (Silver, 1993), and homozygote female sterility of *Sex-Ratio* drive in *Drosophila recens* (Dyer et al., 2007). The accumulation of deleterious mutations is part of the costs that meiotic drivers impose on the rest of the genome. The selfishness leads to a reduction in the productivity of the whole organism, while only the selfish allele and the alleles linked to it benefit from the distorted transmission. The rest of the genome that is unlinked to the driver will thus attempt to suppress the selfish action of the driver (Charlesworth & Hartl, 1978).

Suppression of meiotic drive

The most straightforward way to suppress meiotic drive is to genetically alter the molecular mechanism that allows drive to operate. Indeed, there are many known examples for genetic suppression of drive (Vaz & Carvalho, 2004; Bastide et al., 2011). However, genetic suppression is by no means universal, and a number of drive systems appear to not have evolved genetic suppression despite a long evolutionary history (Burt & Trivers, 2006; Lindholm et al., 2016). Moreover, even when genetic suppression evolves, there will be an ongoing evolutionary arms race between drive and suppression (Bastide et al., 2013). There may be alternative ways to suppress drive that are based on the behaviour of the host organism. One such alternative way is offered if sexual selection allows for discrimination against individuals carrying a driver.

Meiotic drive frequently targets males. This may be in part due to the fact that sex chromosome drive is particularly common in the well-studied Diptera (Jiggins et al., 1999). Particularly *Drosophila* species are genetic model organisms that have long been kept in the lab,

and have male heterogametic sex determination. This may offer a target for sex chromosome drive in males and increased opportunity for researchers to detect it (Jiggins et al., 1999). Additionally, drivers that target male gametogenesis may be more frequent because males of most species produce a large excess of gametes, and killing half of them will reduce fecundity less than in females (Taylor & Ingvarsson, 2003). Moreover, the need to produce a large number of male gametes may select for fast cell proliferation that is subject to less cellular policing than the slower production of female gametes (Kleene, 2005; Lewis et al., 2008). Evidence for haploid gene expression in early spermatids and spermatozoa is accumulating rapidly (Joseph & Kirkpatrick, 2004), demonstrating that spermatogenesis is not always controlled by the diploid genome (Braun et al., 1989). More spermatogenesis genes with haploid expression are likely to be identified in the near future, and these will be promising candidates for identifying new male drive systems or molecular signals of historic drive.

When drive targets males, fertilisation by males that carry the driver can be expected to be costly to females. First, if the driver is located on a sex chromosome, females mating with drive males will produce sex-biased broods. When the population sex ratio is distorted, these females will produce the more common and thus costlier sex (Fisher, 1930). Second, the process of gamete interference can decrease the fertility of male drive carriers, which may in turn threaten female fecundity (Price & Wedell, 2008). Third, the reduced recombination often associated with drive systems leads to the accumulation of deleterious mutations that have negative fitness consequences for the female's offspring. To maximise individual fitness, females should thus avoid fertilisation by drive-bearing males (Wedell & Price, 2015).

Sexual selection and meiotic drive

Sexual selection operates through competition between members of the same sex to produce offspring (Andersson, 1994). Sexual selection is a pervasive evolutionary force that can drive rapid diversification between the sexes and between species. Traditionally, the differences in the reproductive potential between males and females (Darwin, 1871; Bateman, 1948) and in parental care (Trivers, 1972) have been used to explain why males should mate indiscriminately, while females should exert mate choice. More recently, it has been realised that females in the majority of species often mate with more than a single male during a reproductive episode (Taylor et al., 2014). A consequence of female multiple mating (polyandry) is that sperm of more than one male will compete over fertilisation of a given set of ova (sperm competition; Parker, 1970). A male's reproductive success is thus not only determined by his access to mates, but also by the fecundity that he gets per mate (Møller, 1998). Polyandrous females may not only choose mating partners but may also bias fertilisation towards certain males (cryptic female choice; Eberhard, 1996) via a number of different mechanisms (Eberhard, 2009). Males thus have to invest not only into features that make them attractive mating partners, but also into features that ensure successful fertilisation after insemination. This greatly expands the arena for sexual selection by female mate choice, because many mechanisms of precopulatory sexual selection have their analogous mechanism in postcopulatory sexual selection (Birkhead & Moller, 1998).

Choosing mates and/or mating with multiple males are likely to be costly. Increased time spent searching and mating is energetically costly, may trade-off with foraging, and may increase exposure to predation and pathogens (Jennions & Petrie, 2000). Rejecting mates may be risky if future mating opportunities and the quality of future mates are unknown. In order for a reproductive

strategy to pay-off, the benefits of a strategy must outweigh its costs. Many potential benefits of mate choice have been proposed and tested (Andersson, 1994; Andersson & Simmons, 2006). Among these, female discrimination against males carrying meiotic drivers has only recently started receiving attention (but see Lenington, 1983). As highlighted earlier, intra-genomic conflict is a ubiquitous feature of life, and meiotic drive commonly targets male gametogenesis with fitness consequences for females. Meiotic drive may thus represent an underappreciated benefit of pre- and postcopulatory female choice (Price & Wedell, 2008; Wedell & Price, 2015).

The cryptic nature of male drive might make it difficult for females to detect drive-bearing males prior to mating. To achieve drive, targeting spermatogenesis can be sufficient. As a consequence, the only phenotypic difference between drive-free and drive-bearing males may be seen in features of their ejaculate, such as the number and/or motility of transferred sperm. In fact, evolutionary stable precopulatory female preference relies on the presence of a precopulatorily detectable male signal that is tightly genetically linked to the drive locus (Lande & Wilkinson, 1999; Reinhold et al., 1999; Manser, 2015). This is because with recombination, even an initially linked signal will not remain an honest indicator of drive-bearing males. Indeed, in stalk-eyed flies, one of very few examples for female discrimination against drive-bearing males, the target of female preference has been shown to be genetically linked to the drive phenotype (Johns et al., 2005). Females that choose to mate with males with long eye stalks can avoid thus producing heavily female-biased broods (Wilkinson et al., 1998; Cotton et al., 2014). However, the available evidence suggests that precopulatory discrimination is not a common female strategy for avoiding fertilisation by drive-bearing males (Jaenike, 2001; Price & Wedell, 2008; Price et al., 2012). In contrast to precopulatory female choice, postcopulatory sexual selection could simply exploit the fact that drive mechanisms in males sabotage a large proportion of the sperm pool of drive-bearing males (Haig & Bergstrom, 1995). As a consequence of within-ejaculate competition, drive-bearing males' ejaculates may be less competitive in between-ejaculate competition (Haig & Bergstrom, 1995; Zeh & Zeh, 1996; Lorch & Chao, 2003). There is ample empirical evidence from invertebrates that sex chromosome drive reduces sperm competitiveness (Wilkinson & Fry, 2001; Atlan et al., 2004; Price & Wedell, 2008; Price et al., 2008a) and thus that polyandry can be an effective counterstrategy against male drive (Price et al., 2008b). There are some indications that this is true for autosomal drive, too, but the empirical evidence is less compelling (Olds-Clarke & Peitz, 1985; Ardlie & Silver, 1996; Manser et al., 2011). Postcopulatory sexual selection has another big potential advantage over precopulatory selection. While precopulatory mate choice is constrained to selecting the ideal mate based on his diploid genotype, postcopulatory selection offers the potential to select at the haploid stage even within an ejaculate, and thus to optimise offspring genotypes (e.g., when genetic fitness effects are non-additive; Puurtinen et al., 2009). In the context of drive-bearing males, this might allow females to select non-drive-bearing spermatozoa for fertilisation of their ova. That is unless wild type sperm are already killed during spermatogenesis and are excluded from the ejaculate.

A number of features that are outlined above make the drive mechanism of the *t* haplotype in house mice a particularly promising candidate for drive suppression through sexual selection.

The *t* haplotype in house mice

The *t* haplotype is a spectacular example of how fair Mendelian segregation can fail. Strong male drive and deleterious recessive mutations create a strong incentive for females to avoid

fertilisation by male carriers of the *t* haplotype. To understand how sexual selection might resolve genomic conflict, we need to start with how the *t* haplotype drives and how that affects the fitness of its host species. In the following section, I review how the *t* haplotype achieves strong drive in males. While much is known about the molecular basis of drive, less is understood about what regulates *t* frequencies in wild house mouse populations. In subsequent sections, I discuss the evolutionary forces that may explain *t* frequencies, and will briefly outline why sexual selection represents a particularly promising evolutionary force for determining the fate of the *t* haplotype in the wild. These arguments will be elaborated further in the individual data chapters.

Drive mechanism

The *t* haplotype is a large stretch of DNA located on the mouse chromosome 17 that remains connected through major chromosomal inversions that prevent recombination through crossing-over (Hammer et al., 1989). A number of the many alleles that the *t* haplotype encompasses contribute to post-meiotic drive in males (for reviews see Lyon, 2003; Presgraves, 2009; Herrmann & Bauer, 2012). A number of different *t* haplotype variants have been described and categorised into 16 complementation groups (Klein et al., 1984), but all extant *t* haplotypes share a common evolutionary history (Silver, 1993) and the basic mechanism of drive is thought to be common to all *t* haplotypes.

Meiosis in males that are heterozygous for the *t* haplotype (+/*t* males) results in equal numbers of spermatids that carry the *t* haplotype and the wild type (+) homologue (Silver & Olds-Clarke, 1984). During spermatogenesis however, spermatids containing the *t* haplotype postmeiotically express *t*-specific distorter genes whose products are shared among all spermatids through cytoplasmic bridges, which is thought to be common for gene products involved in mammalian spermatogenesis (Braun et al., 1989; Ventelä et al., 2003). Although only some of these distorter genes have been identified (Bauer et al., 2005, 2007), early studies on recombinant *t* haplotypes that lack some of the inversions demonstrated that at least four distorters with additive action are spread over different inversions (Lyon, 1984). The products of these distorters have detrimental effects on a sperm motility kinase that regulates sperm movement (Herrmann et al., 1999). However, the gene encoding this sperm motility kinase is located in the genomic region of the *t* haplotype, which thus possesses its own *t*-specific kinase that is a functional hypomorph of the wild type kinase (Herrmann et al., 1999). Importantly, the kinase (the 'responder' in the drive system) can act in *cis* because it is retained in the cell of production instead of being shared through the cytoplasmic bridges (Véron et al., 2009). As a consequence, sperm that do not contain the *t* haplotype have an impaired fertilisation potential (Olds-Clarke & Peitz, 1985).

Distorters and responder can be compared to 'poison' and 'antidote'. During an early stage, the 'posion' (i.e., the distorters) is distributed among all spermatids. It is only later that the 'antidote' (i.e., the responder) is produced, which is retained in the cells that produce it. Thus, the 'antidote' locally counteracts the global action of the 'poison'. Within a single ejaculate, many millions of sperm are released, but there are only a few ova available for fertilisation. In mammals, sperm motility is an important determinant of reaching the site of fertilisation and penetrating the ova vestments (Suarez, 2008b), and sperm motility appears to critically depend on the optimal regulation of sperm motility kinases. Although +/*t* males ejaculate equal numbers of + and *t* sperm (Silver & Olds-Clarke, 1984), the + sperm are not protected from the negative effects of the distorters on motility, and their motility is moved away from the optimum (Katz et al., 1979; Olds-Clarke & Johnson, 1993) which results in a lower success in intra-ejaculate competition (Olds-Clarke &

Peitz, 1985; Olds-Clarke, 1997). As a consequence, + sperm only fertilise a small minority, while *t* sperm fertilise the vast majority of the ova (up to 99%; Chesley & Dunn, 1936; Dunn, 1957; Bennett et al., 1983; Lindholm et al., 2013). Because the *t* haplotype affects postmeiotic spermatogenesis, *t* heterozygous females transmit the *t* haplotype in Mendelian ratios (Chesley & Dunn, 1936; Ardlie & Silver, 1996; Lindholm et al., 2013).

t haplotype frequencies in wild populations

The *t* haplotype's strong drive in +/*t* males drive would lead to rapid fixation of the *t* haplotype in a population if there were no opposing evolutionary forces (Burt & Trivers, 2006). However, one important consequence of the additive action of the *t*-specific distorters in *trans* is that males homozygous for the *t* haplotype (*t/t*) have an excess of distorter gene products that is not sufficiently counteracted by the reduced sensitivity of the *t*-specific kinase hypomorph. As a result, all sperm of a *t/t* male have drastically reduced motility that results in male sterility (Lyon, 1986). Consequently, the *t* haplotype cannot propagate through *t/t* males, while *t/t* females are fertile. Given male homozygous sterility, fixation of the *t* haplotype in natural populations would inevitably result in population extinction (Lewontin, 1962).

Homozygote lethality is an additional force that prevents the fixation of the *t* haplotype in most wild populations. The integrity of the *t* haplotype is ensured by four major chromosomal inversions, providing strong physical linkage and thus co-inheritance of the multiple distorter genes and the *t*-specific responder (Hammer et al., 1989). Many *t* haplotype variants have lethal effects during early embryogenesis (Bennett, 1975; Safronova, 2009), possibly as a direct consequence of a lack of recombination with wild type homologues and subsequent accumulation of recessive deleterious mutations. Interesting alternative views invoke group and kin selection arguments to explain how early embryo lethality may be adaptive rather than a mere by-product of mutation accumulation (Dunn & Levene, 1961; Charlesworth, 1994). Because *t/t* males are sterile, they represent an evolutionary dead-end to the *t* haplotype. From a meta-population perspective, demes with lethal alleles may benefit from a lowered extinction risk by avoiding *t* fixation and male fertility failure (Dunn & Levene, 1961; Lewontin, 1962, 1968). The kin selection perspective argues that if early embryo lethality benefits other *t* bearing sibling in utero, the fitness loss through lethality could be compensated for by increased sibling survival or competitiveness (Charlesworth, 1994). However, considering the loss of fertile *t/t* female offspring, the compensatory effects would need to be substantial for this lethality to be adaptive, unless *t/t* daughters are also of reduced fertility (Burt & Trivers, 2006). While these are interesting hypotheses, they may be less plausible than the view based on accumulation of deleterious mutations (Presgraves, 2009).

It is clear that once homozygote lethality is established, the *t* haplotype cannot reach fixation. Still, models that have used strong male drive and homozygote lethality to predict frequencies in wild populations (Bruck, 1957) yield strong overestimations. *t* frequencies in wild house mouse populations are typically much lower than predicted (Klein et al., 1984; Figueroa et al., 1988; Lenington et al., 1988b; Ruvinsky et al., 1991; Ardlie & Silver, 1996, 1998; Huang et al., 2001; Dod et al., 2003; Ben-Shlomo et al., 2007; Baker, 2008; Safronova et al., 2010; Manser et al., 2011). The discrepancy between observed and expected frequencies has been termed the '*t* frequency paradox', and a number of attempts have been made to explain it (reviewed in Ardlie, 1998).

Resolving the t frequency paradox?

With empirical evidence from wild populations accumulating, it became clear that there must be important evolutionary forces acting in the wild in addition to drive and homozygote lethality. Because the original models had assumed large panmictic populations (Bruck, 1957), and processes that increase homozygosity such as inbreeding (Petras, 1967) should have negative effects on *t* haplotype frequencies, population subdivision was invoked as a possible resolution to the *t* frequency paradox. Drift would become more important if populations consisted of small and isolated demes (Lewontin & Dunn, 1960; Levin et al., 1969). Curiously, the *t* haplotype is typically absent from strongly isolated populations such as those on islands (Dunn et al., 1960; Myers, 1973), possibly indicating that a reduction in migration decreases *t* frequencies. However, empirical estimates of inter-deme migration rates in wild populations appear too high for deme structure to solely explain the *t* frequency paradox (Nunney & Baker, 1993; Durand et al., 1997; Ben-Shlomo et al., 2007). The empirical observation that *t* frequencies are lower in large than in small populations has nonetheless suggested that population structure might play a role, possibly through density-dependent effects (Ardlie & Silver, 1998; Baker, 2008).

What the models seemed to require was an additional disadvantage to the *t* haplotype, such as selection against heterozygotes (Young, 1967) or reduced drive in wild populations. Some genetic modifiers were detected in lab populations (Bennett et al., 1983; Gummere et al., 1986), and modifiers could similarly reduce drive in wild populations. However, genetic modifiers were ruled out as a general explanation for low *t* frequencies in wild populations, because drive estimates for wild +/*t* males were high based on wild-caught pregnant females and controlled lab matings using wild-caught mice (Silver, 1985; Ardlie & Silver, 1996). Reduced drive was also detected when mating in the lab was experimentally delayed (Braden, 1958) and when litters were conceived in postpartum oestrus (Lenington & Heisler, 1991). However, the effect of mating delay on drive could only be confirmed for some, but not other *t* haplotype variants (Yanagisawa et al., 1961), and an additional study on the effect of oestrus mode on drive found no difference between litters conceived during cycling versus postpartum oestrus (Ardlie & Silver, 1996). Reduced drive is thus an unlikely explanation for low *t* frequencies. The fitness of *t* heterozygous mice has been extensively studied, mostly in the lab but also in wild populations. The evidence for differences in viability, social dominance and fertility and fecundity is mixed. Survival estimates were equal for +/*t* and +/+ individuals in some studies (Ardlie & Silver, 1998; Huang et al., 2001), while others found that +/*t* had lower (Carroll et al., 2004), or higher survival rates (Dunn et al., 1958; Manser et al., 2011). Similarly, social dominance of +/*t* males was found to be higher in some studies (Franks & Lenington, 1986; Lenington et al., 1996) but not in another (Carroll et al., 2004). There is a generally consistent pattern that litters are smaller when one of the parents is +/*t* (Johnston & Brown, 1969; Lenington et al., 1994; Ardlie & Silver, 1996; Carroll et al., 2004; Lindholm et al., 2013), though the differences are not always significant and are too small to resolve the *t* frequency paradox. Nevertheless, lowered fecundity of +/*t* individuals is a likely candidate for explaining low *t* frequencies in natural populations, especially if there is an additional disadvantage in mating success or competitive fertilisation success.

Sexual selection and the t haplotype

In a laboratory study on male-male competition between +/*t* and +/+ males, Levine et al. (1980) found that 67% of the offspring were sired by +/+ males, and concluded that +/*t* males must be disadvantaged either in pre- or postcopulatory sexual selection. In a number of experiments,

Lenington and colleagues subsequently investigated female preference for +/+ over +/*t* males and repeatedly found that +/*t* females preferred to associate with +/+ males or their scent over +/*t* males or their scent (Lenington, 1991; see Chapter 3). The argument for why +/*t* females should avoid fertilisation by +/*t* males is compelling, as +/*t* individuals are genetically incompatible. As a direct result of strong male drive and *t*/*t* lethality, +/*t* females lose around 40% of their embyros if they are sired by a +/*t* male (Bennett, 1975; Lindholm et al., 2013). Because female investment in gestation and lactation is costly, females should avoid fertilisation by +/*t* males. Reduced fertility of +/*t* males may represent an additional fitness cost to all females irrespective of their genotype at the *t* locus. Detection of +/*t* males through olfaction could be mediated by the major histocompatibility complex (MHC), of which some alleles are located in the genomic region of the *t* haplotype. *t* haplotypes are associated with unique MHC alleles (Artzt, 1986; Ben-Shlomo et al., 2007; Lindholm et al., 2013), offering a target that females could use for discrimination. However, female preference had never been measured in an actual mating context, and male dominance appeared to override the effects of the *t* haplotype on olfactory preferences (Lenington, 1991). Additional reports from semi-wild and wild populations found some indications for an effect of sexual selection on +/*t* male reproductive success (Carroll et al., 2004; Lindholm et al., 2013), but were based on paternity analyses and thus unable to identify the stage at which selection had operated. Using an elaborate choice apparatus that allowed females to freely move between males Manser *et al.* (2015) found no evidence for female social preference for +/+ males. Nonetheless, paternity data showed that reproductive success was lower for +/*t* males, and that +/*t* males obtained a smaller paternity share than +/+ males in litters with mixed paternity. Though based on a small sample size, this result was in line with previous reports that suggested +/*t* males were disadvantaged (Olds-Clarke & Peitz, 1985; Ardlie & Silver, 1996; see Chapter 1). Assuming a sperm competitive disadvantage to +/*t* males, a modelling approach showed that polyandry alone could account for the *t* frequency decline observed in a closely monitored wild population (Manser et al., 2011). The increased intra-ejaculate competition in +/*t* ejaculates may well have negative effect on inter-ejaculate competitiveness, and polyandrous females may thus benefit from multiple mating if it increases the chances of fertilisation by +/+ males.

Data chapters: Sexual selection experiments

In the five main chapters of this thesis, I report results from controlled laboratory experiments that addressed the following questions:

- Do females actively discriminate against +/*t* males?
- How does the *t* haplotype affect sperm competition?
- Do females benefit from multiple mating by avoiding fertilisation by +/*t* males?
- How do males adjust their mating strategies to maximise their paternity success?

The individual chapters address how polyandry affects the relative reproductive success of +/*t* males and the consequences for female fitness (Chapter 1), how *t*-specific sperm precedence may have consequences for male reproductive strategies (Chapter 2), whether females actively discriminate against +/*t* males at pre- and postcopulatory stages (Chapter 3), and how the copulatory plug may benefit male mice in sexual selection (Chapters 4 & 5).

CHAPTER 1 DETRIMENTAL EFFECTS OF AN AUTOSOMAL SELFISH GENETIC ELEMENT ON SPERM COMPETITIVENESS IN HOUSE MICE

1.1 ABSTRACT

Female multiple mating (polyandry) is widespread across many animal taxa and indirect genetic benefits are a major evolutionary force favouring polyandry. An incentive for polyandry arises when multiple mating leads to sperm competition that disadvantages sperm from genetically inferior mates. A reduction in genetic quality is associated with costly selfish genetic elements (SGEs), and studies in invertebrates have shown that males bearing sex ratio distorting SGEs are worse sperm competitors than wild type males. We used a vertebrate model species to test whether females can avoid an autosomal SGE, the *t* haplotype, through polyandry. The *t* haplotype in house mice exhibits strong drive in *t* heterozygous males by affecting spermatogenesis and is associated with homozygous *in utero* lethality. We used controlled matings to test the effect of the *t* haplotype on sperm competitiveness. Regardless of mating order, *t* heterozygous males sired only 11% of zygotes when competing against wild type males, suggesting a very strong effect of the *t* haplotype on sperm quality. We provide, to our knowledge, the first substantial evidence that polyandry ameliorates the harmful effects of an autosomal SGE arising through genetic incompatibility. We discuss potential mechanisms in our study species and the broader implications for the benefits of polyandry.

Key words: t haplotype, segregation distortion, polyandry, embryo viability, indirect benefits, genetic incompatibility

1.2 Introduction

When females mate with multiple males, sperm from different males compete for fertilization of the ova (Parker, 1970). By inciting sperm competition, females may prolong male–male competition beyond pre-copulatory contest and bias fertilization towards males of high quality or compatibility (Jennions & Petrie, 2000), with major effects on sexual behaviour, sex allocation, social networks, sexually transmitted infections, population viability and speciation (Pizzari & Wedell, 2013). Despite the many demonstrations of direct and indirect (genetic) benefits of polyandry (Jennions & Petrie, 2000), there is still no real consensus on why polyandry is so ubiquitous in nature.

One possibly underappreciated benefit of polyandry is protection from costly selfish genetic elements (SGEs) driving through males (Wedell, 2013). SGEs are sequences that alter DNA replication in their own favour, increasing their representation in the subsequent generation (called drive or segregation distortion) at the cost of their homologous sequences and usually also of the rest of the genome (Burt & Trivers, 2006). SGEs that kill or interfere with gametes carrying the homologous gene or chromosome, called gamete killers (Burt & Trivers, 2006), typically drive through males. This is presumably because male gametes are produced in excess so that destruction of gametes has a smaller effect on fertility in males than in females (Taylor & Ingvarsson, 2003). Driving elements can occur on sex chromosomes or on autosomes, but sex chromosome drive is expected to arise more easily than autosomal drive (Hurst & Pomiankowski, 1991). However, modifiers of sex chromosome drive are strongly selected for because mating with a driving male will result in a costly single sex brood (Hurst & Pomiankowski, 1991). Given the relative amount of information encoded on autosomes versus sex chromosomes, more genomic regions may be available in which novel autosomal drivers can evolve. In addition, autosomal drive is much less likely to be detected because of the lack of sex-biased broods (Taylor & Ingvarsson, 2003). Consequently, there has been a detection bias towards sex ratio distorting SGEs (Taylor & Ingvarsson, 2003). Indeed, autosomal drive has so far mainly been studied in model systems, such as mice (*t* haplotype; Silver, 1985), and *Drosophila* (*Segregation Distorter;* Sandler et al., 1959). Thus, autosomal drive through males may be more common than observed, but the relative importance of autosomal versus sex chromosomal drive for evolution within the genome remains unclear. As whole genome scans become increasingly common, more SGEs are likely to be identified (Casellas et al., 2012).

Male drivers can be expected to incur fitness disadvantages. Male-driving autosomal SGEs are associated with inferior genetic quality, the most extreme costs arising through recessive lethal mutations or sterility in homozygous carriers (Hartl et al., 1967; Zeh & Zeh, 1996; Tregenza & Wedell, 2000). In heterozygous males, SGE bearing sperm harm their wild type bearing counterparts and ensure the SGE's transmission to a large proportion of the offspring (Burt & Trivers, 2006). In SGE homozygous males however, sperm bearing homologous copies of the SGE can render each other dysfunctional, leading to strong fertility reduction or even sterility (Hartl, 1969; Lyon, 1986). Despite strongly deleterious effects of reduced male fertility or homozygous lethality, autosomal SGEs can be maintained in populations through drive (Hartl, 1970). Females thus face the risk of mating with males of inferior genetic quality with negative effects on the number and genetic quality of their offspring.

When the drive mechanism involves killing or harming sperm not carrying the SGE during spermatogenesis, polyandry can be an effective means of avoiding carriers of SGEs because as a direct consequence of drive, these males have fewer viable or functional sperm (Haig & Bergstrom, 1995; Zeh & Zeh, 1996; Lorch & Chao, 2003; Price & Wedell, 2008). Indeed, reduced sperm competitiveness of males carrying SGEs has been reported in stalk-eyed flies and several *Drosophila* species (Wu, 1983; Wilkinson & Fry, 2001; Atlan et al., 2004; Price et al., 2008a). Further support comes from studies reporting associations between female remating rate and sex ratio distorting chromosomes across wild populations of *Drosophila* and stalk-eyed flies (Wilkinson et al., 2003; Pinzone & Dyer, 2013; Price et al., 2014). Empirical evidence for the effect of polyandry on autosomal SGEs is however very scarce (Price & Wedell, 2008). Here, we investigated the influence of an autosomal SGE on postcopulatory sexual selection in a vertebrate.

The *t* haplotype in house mice is a very intensively studied SGE (Burt & Trivers, 2006). Typically, *t* haplotypes are inherited by 90% of the offspring of male carriers (denoted as $+/t$) and by 50% of offspring of female carriers, but t/t offspring perish *in utero* owing to recessive lethal mutations (Klein et al., 1984; Lindholm et al., 2013). Immediate fitness costs associated with the *t* haplotype are thus related to genetic incompatibility: $+/t$ females mated to $+/t$ males have 40% smaller litters than when mated to $+/+$ males (Lindholm et al., 2013). $+/t$ females are predicted to avoid this strong cost of genetic incompatibility associated with fertilization by $+/t$ males. There is ample empirical evidence that sexually receptive $+/t$ females prefer the odour and the proximity of $+/+$ males over $+/t$ males (Lenington et al., 1992). However, $+/+$ females might also benefit from avoiding fertilization by $+/t$ males if the *t* haplotype also exhibits additive detrimental fitness effects, but the evidence so far is mixed (e.g., behavioural dominance: Lenington et al., 1996; Carroll et al., 2004).

The basis for the *t* haplotype's selfishness—arguably its main effect—is its impact on spermatogenesis. Drive in $+/t$ males is due to an elaborate molecular mechanism resulting in abnormal flagellar function of + sperm, comparable to a 'poison–antidote' system (Herrmann & Bauer, 2012). This is predicted to have an effect on sperm competitiveness of $+/t$ males through a numerical reduction of functional sperm. To achieve a drive of 90%, most + sperm in a $+/t$ male's ejaculate are rendered dysfunctional, reducing the number of functional sperm by about 45%. Although $+/t$ males have the same number of epididymal sperm as $+/+$ males, their sperm show reduced velocity and linearity and importantly, fewer sperm at the site of fertilization (reviewed in Olds-Clarke, 1997). In monogamous matings, fertility of $+/t$ males tends to be lower than that of $+/+$ males (Carroll et al., 2004; Lindholm et al., 2013). Thus, $+/t$ males probably ejaculate fewer functional sperm. However, the effect on the inter-ejaculate competitive ability of the remaining functional sperm remains unknown. Indications for reduced sperm competitive abilities of $+/t$ males are restricted to few studies using very small sample sizes (Olds-Clarke & Peitz, 1985) and which did not use controlled matings. Assuming a fair raffle model where the number of functional sperm corresponds to the number of tickets bought in a lottery (Parker, 1990), the predicted paternity share of $+/t$ males is about 35% owing to the reduction in functional sperm numbers.

Here, we used many experimental matings to investigate: (i) sperm competitiveness of $+/t$ males, (ii) fitness consequences for polyandrous females in the form of embryo viability, and (iii) whether $+/t$ and $+/+$ males invest differentially into sperm production.

1.3 Material and Methods

1.3.1 Experimental animals

We used 90 male and 140 female laboratory-born house mice (*Mus musculus domesticus*), F1 to F3 descendants from a free-living population of wild house mice in Switzerland (König & Lindholm, 2012). At every generation, we introduce mice from the free-living population into our breeding colony. Laboratory conditions were a reversed 14L:10D cycle (lights on at 17.30) and a temperature of 22–24°C. Food (mouse and rat breeding diet, Provimi Kliba AG) and water were provided ad libitum, paper towels and cardboard served as enrichment and nest building material. Breeding pairs consisted of monogamously paired non-sibling +/+ males and +/*t* females, producing on average 50% +/*t* offspring. Offspring were weaned at 28 days after birth and kept in same sex sibling groups in Macrolon Type III cages (425 × 266 × 155 mm). We used +/*t* and +/+ males and females and diagnosed their *t* haplotype status before they entered the experiment. An ear punch taken at weaning was used for genotyping and individual marking. *t* haplotype status was diagnosed by PCR (Schimenti & Hammer, 1990; Lindholm et al., 2013). Male mice were separated latest at the onset of aggression between brothers and kept individually in Macrolon Type II cages (180 × 240 × 140 mm). The experimenter was blind with respect to the mice's *t* genotype during all procedures, including mating trials, female and male dissections, and video observations (see below).

1.3.2 Sperm competition trials

For our experimental matings, we followed a protocol modified after (Firman & Simmons, 2008a). Details on mating design and paternity assignment are given in Supplement A. Briefly, we conducted sperm competition trials using full brother pairs differing in *t* haplotype genotype by mating them to virgin +/+ and +/*t* females in cycling oestrous. By using full brothers, we largely controlled for potential effects of genetic background and maternal environment on sperm competitiveness. We conducted up to four trials to balance mating order (as there is first male precedence in house mice; Firman & Simmons, 2008a) and female *t* genotype. During mating trials, pairs were checked every 1–1.5 h for copulatory plugs indicative of ejaculation (Rugh, 1968). Once a copulatory plug was detected, the female was added to the second male's cage and checked every 30–60 min until either a second copulatory plug was observed or until the beginning of the next dark phase. We confirmed and counted ejaculations using video recordings. To obtain unbiased estimates of paternity share (before *t*/*t* embryos are resorbed Lindholm et al., 2013), we sacrificed females 9 days (±1 day) *post coitum* using gradual CO2 filling in their home cage and dissected females to retrieve implanted embryos. We scored 12 microsatellites spread across 10 autosomes and assigned paternity using CERVUS v. 3.0 (Kalinowski et al., 2007).

1.3.3 Embryo viability

To investigate fitness consequences for females, we assessed embryo viability based on developmental stage. At day 9, normal embryos have clearly visible somites and forelimb buds begin to form (Theiler Stages 13 or 14; Theiler, 1989). During dissection, we recorded the number of implantation sites and the development stage of individual embryos. Embryos with normal morphological appearance were classified as viable, whereas embryos with arrested

development (i.e., Theiler Stage 10 or earlier) as well as resorbed embryos were classified as inviable.

1.3.4 Male reproductive organs

As we could not measure ejaculate size directly, we investigated potential *t* haplotype-associated differences in sperm production and storage by weighing testes and epididymides *post-mortem*.

1.3.5 Statistical analyses

Sample sizes available for statistical analyses are summarized in SUPPLEMENT A, Table S1. Of the 140 females used for mating trials, 95 mated after an average of two trials (range 1–12). Seventeen females did not become pregnant, 15 did not mate with the second male and remating could not be unambiguously determined for a further six. Because of our focus on postcopulatory processes, trials without ejaculation by the second male were omitted from further analyses, except for analysis of the effect of $+/t$ paternity share on embryo viability (see below). For 16 of the 57 remaining females, we were not able to unambiguously quantify the number of ejaculations. Thus, our final sample sizes were 41 females (320 out of 329 embryos genotyped) for the effect of ejaculation number on paternity share and 57 females (440 out of 453 embryos genotyped) for the other variables.

All statistical analyses were performed in R, v. 3.0.2 (R CORE TEAM, 2015). We analysed *t* paternity share with generalized linear mixed models (GLMMs), using the function glmer in lme4 (BATES ET AL., 2014). The number of embryos sired by the $+/t$ male was included as the dependent variable and the number of embryos genotyped for a given female as the binomial denominator. Mating order, female *t* genotype, the relative difference in body weight between the competing males and the difference in the number of ejaculations of the $+/t$ versus $+/+$ male were fitted as fixed effects with biologically relevant two-way interactions. To avoid pseudo-replication, we included male pair as a random factor. We accounted for overdispersion by including an observation-level random effect and compared models based on the Akaike information criterion corrected for small sample sizes (AICc) using the dredge function in MuMIn (BARTOŃ, 2015). To get estimates and confidence intervals (CIs), we back-transformed best model estimates from the logit to the original scale. We obtained approximate 95% confidence intervals by multiplying Student's t-values for our sample sizes by standard errors of the predicted values before back-transformation to the original scale (CRAWLEY, 2007).

The proportion of viable embryos was analysed in analogy to *t* paternity share, using 57 polyandrous females (446 out of 453 embryos classified for viability) and 15 monandrous females (122 out of 124 embryos classified for viability). The delay between mating and dissection did not have an effect on embryo viability and was not included in subsequent models. To test for a benefit of a reduction in $+/t$ paternity share on embryo viability, $+/t$ male paternity share, the female's genotype and an interaction between $+/t$ male paternity share and female genotype were included as fixed effects. Female body weight was included as an additional fixed effect and male pair was included as a random effect.

We analysed testes and epididymides weights with linear models and log-transformed organ and body weight to achieve normality of residuals. Full models included *t* genotype, body weight and its interaction as fixed effects. We selected the minimal adequate model using stepwise backwards model selection based on log-likelihood.

1.4 Results

1.4.1 +/t paternity share

Female *t* genotype was not retained during model selection for +/*t* paternity share analysis and females were hence pooled. In 57 trials of polyandrous females, +/*t* males sired only 57 of the 440 embryos genotyped (12.9%). The GLMM including mating order and the relative weight difference between males performed best as indicated by the lowest AICc value. Here, mating order (z =-4.11, n = 55, P < 0.001) and body weight difference (z = 4.04, n = 55, P < 0.001) had significant effects on paternity share, but +/+ and +/*t* males did not differ in body weight (ANOVA, $F_{1,74}$ = 0.12, P = 0.731). When mating first, +/*t* males sired 21.7% of the offspring as opposed to 4.7% when mating second. The model prediction for mean +/*t* male paternity share was 11.3% (approx. 95% CI 6.2–19.6%; left chart in Figure 1.1). This strongly differs from the null hypothesis of equal paternity share between +/*t* and +/+ males (dashed grey line in Figure 1.1; z =-4.33, n = 55, P < 0.001). Notably, the upper confidence level of the +/*t* paternity share was also well below the adjusted null hypothesis, predicted by the reduction in the number of functional sperm through drive. With 90% drive by the *t* allele (previously measured in Lindholm et al., 2013), the majority of + sperm are rendered dysfunctional and are not competitive against other males' sperm (predicted +/*t* paternity share 35%, solid grey line in Figure 1.1). We obtained an estimate of male drive from 37 embryos sired by a +/*t* male mated to a +/+ female. Thirty-one out of 37 (84%) embryos paternally inherited the *t* haplotype, not significantly different from 90% ($\chi^2(1)$ = 1.59, P = 0.208). In the reciprocal cross, 60 out of 125 (48%) embryos maternally inherited the *t*, not different from Mendelian segregation ($\chi^2(1)$ = 0.2, P = 0.655).

1.4.2 Ejaculation frequency

During video analysis, we found that males ejaculated twice between two cage checks in some of the trials. Thus, in our second model selection approach, we included only trials for which we knew the exact number of ejaculations by both males. The model including only the difference in number of ejaculations between competitors received strongest AICc support. An additional ejaculation by the +/*t* male enhanced his paternity share to 45% (right chart in Figure 1.1; GLMM: z = 3.895, n = 41, P < 0.001). In 11 out of 41 trials, the first male to mate ejaculated twice, whereas the second male ejaculated twice in only 1 out of 41 trials. Thus, when accounting for the number of ejaculations, neither mating order nor body weight had a significant effect on paternity share. Ejaculation number was independent of male *t* status, with five +/*t* males and seven +/+ males ejaculating twice (Figure 1.1; $\chi^2(1)$ = 0.05, P = 0.818).

1.4.3 Embryo viability

The model best explaining embryo viability included the interaction between +/*t* male paternity share and female genotype as well as female body weight. Thus, the proportion of viable embryos was significantly influenced by the interaction between +/*t* paternity share and female genotype, i.e., +/*t* females had a lower proportion of viable embryos when +/*t* paternity share increased (top chart in Figure 1.2; GLMM: z = 3.59, n = 70, P < 0.001). Indeed, all 18 embryos that had the *t/t* genotype were inviable. By contrast, only 8 out of 152 (5.3%) of the +/*t* embryos and 15 out of 373 (4.0%) of the +/+ embryos were inviable, respectively. Female body weight at the time of

mating had a positive effect on embryo viability (GLMM: $z = 2.38$, $n = 70$, $P = 0.017$) but body weight did not differ between +/+ and +/*t* females (ANOVA, $F_{1,68} = 0.035$, $P = 0.853$) or between monandrous and polyandrous females ($F_{1,68} = 0.71$, $P = 0.401$).

1.4.4 Male reproductive organs

Both testis and epididymis weight correlated positively with body weight, but there were no differences between +/+ and +/*t* males in body weight (see section *+/t paternity share*). Epididymis weight was slightly more strongly correlated to body weight ($F_{1,74} = 3.99$, $P < 0.001$, $R^2 = 0.12$) than was testis weight ($F_{1,74} = 2.93$, $P = 0.005$, $R^2 = 0.10$). Neither organ showed an association with *t* genotype (testes: $F_{2,73} = -0.68$, $P = 0.502$; epididymis: $F_{2,73} = 0.32$, $P = 0.750$).

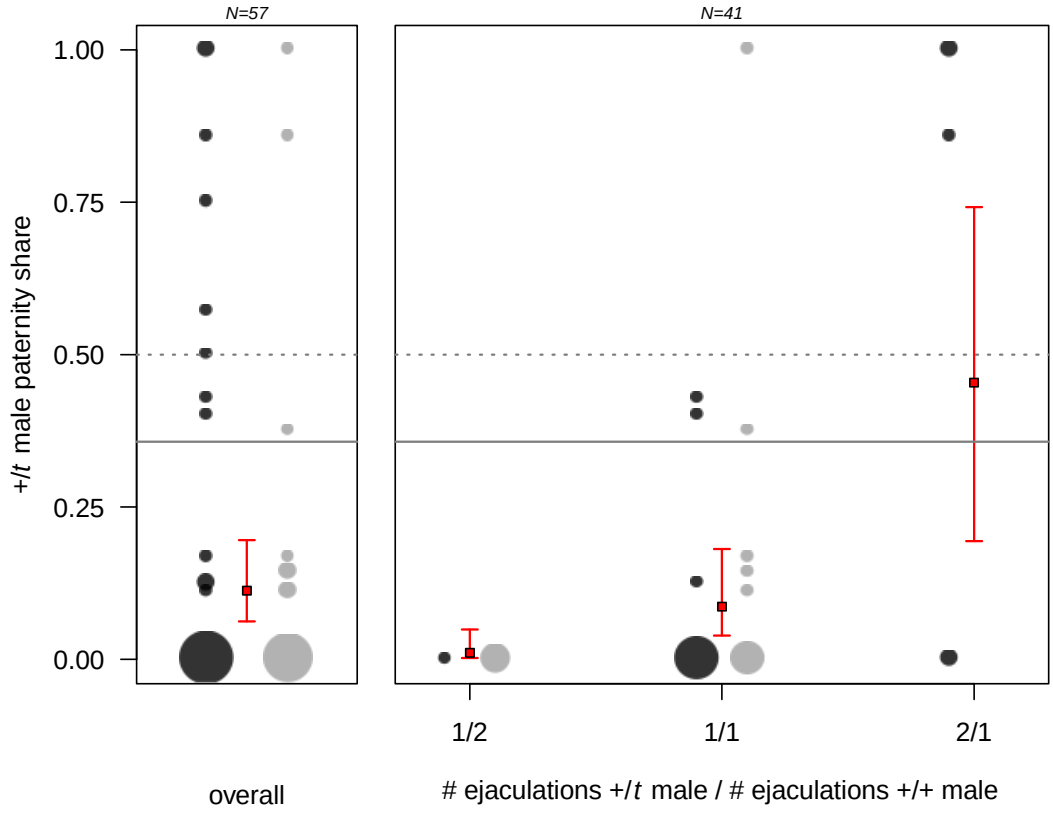

Figure 1.1

Paternity share of +/*t* males in sperm competition with +/+ males. Shown are overall paternity share (left chart) and paternity share as a function of the number of ejaculations (right chart). The surface area of grey circles is proportional to the number of observations. Colours of circles represent mating order, with dark grey indicating trials in which the +/*t* male was first to mate. Mating order did not have a significant effect on paternity share when accounting for the number of ejaculations and is included for illustrational purposes only. Red squares and error bars represent mean and approximate 95% confidence interval estimates. The grey dotted line shows equal paternity share for +/+ and +/*t* males and the grey solid line represents the prediction based on a numerical reduction in functional sperm through drive (see main text).

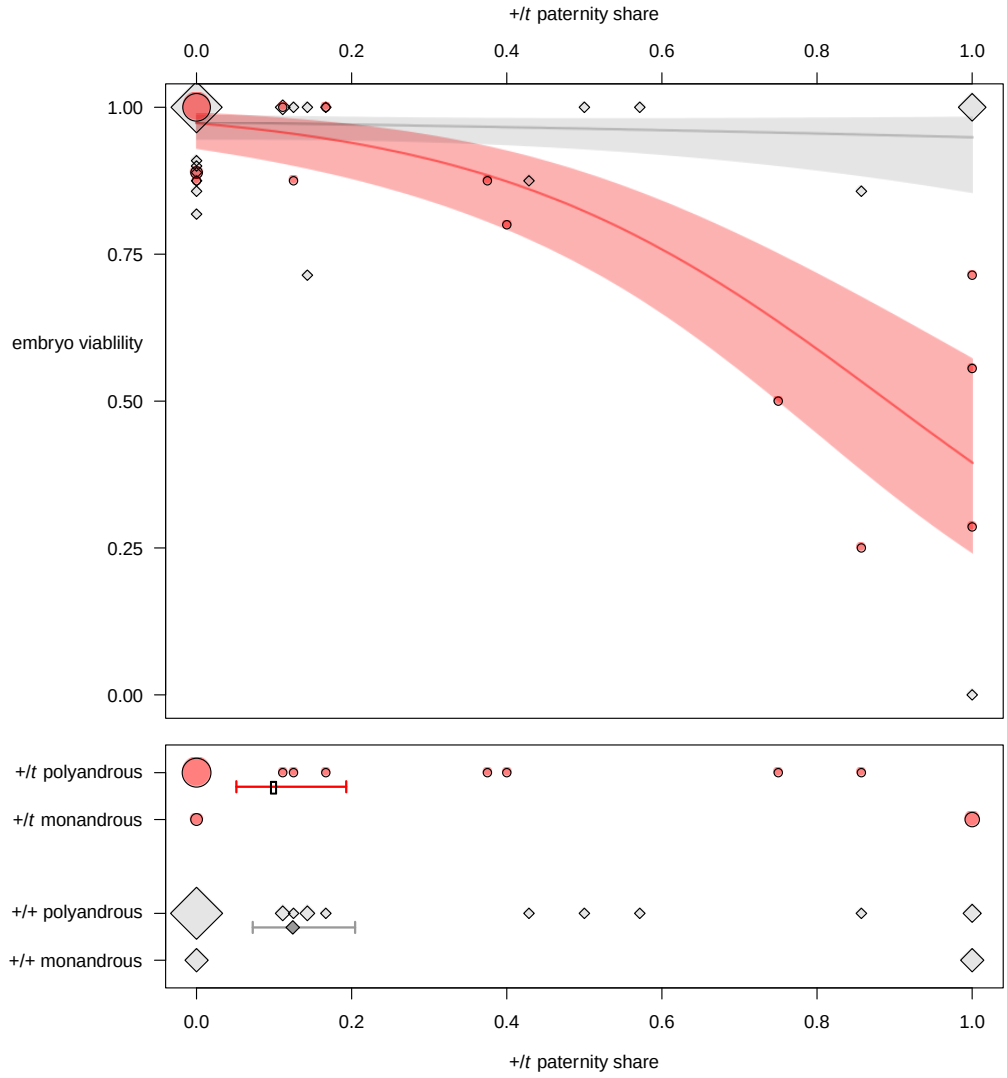

Figure 1.2

+/*t* paternity share in polyandrous and monandrous females (bottom chart) and consequences for embryo viability (top chart). Monandrous females were mated to either a +/*t* or a +/+ male (+/*t* male paternity share 1 and 0, respectively), whereas polyandrous females mated with both a +/*t* and a +/+ male. Colours and shapes indicate female genotype (+/+ in grey diamonds, +/*t* in red circles). The surface area of diamonds and circles is proportional to the number of observations. Mean and approximate 95% confidence interval estimates are indicated by points and error bars (bottom chart) and lines and shaded areas (top chart), respectively.

1.5 Discussion

We show that the *t* haplotype in house mice is associated with a strong disadvantage in postcopulatory competition. +/*t* males sired dramatically fewer offspring than their +/+ brothers, regardless of mating order. This paternity share was significantly lower than the adjusted null hypothesis (35%), which accounts for the effect of drive on the number of functional sperm in a +/*t* male's ejaculate. We further show that this severely reduced paternity share results in an immediate benefit for polyandrous +/*t* females by reducing costly *t*-associated genetically incompatible fertilizations.

1.5.1 Postcopulatory competition

In sperm competition against +/+ males, +/*t* males sired only 11% of a female's implanted embryos. Notably, *t* paternity share was even lower than predicted from the number of functional sperm in a raffle model. If *t* haplotype drive is achieved by harming +sperm alone, then the 90% drive observed in our study population should reflect a decrease in the number of functional sperm by about 45%, providing an adjusted null hypothesis of about 35% *t* paternity share. The upper level of the approximate CI (20%) was well below this prediction. This suggests that not only does drive harm + sperm (Olds-Clarke, 1997), but also damages *t* sperm in +/*t* males. The 'poison–antidote' mechanism favouring *t* sperm within a +/*t* male's ejaculate (for details see Herrmann & Bauer, 2012) thus appears to be imperfect insofar as it results in a strong between-ejaculate disadvantage when a +/*t* ejaculate competes against a +/+ ejaculate. Thus, the *t* haplotype's 'antidote' does not appear to provide full protection from the *t* haplotype's own 'poisonous' effect. Previous experimental evidence for a +/*t* male sperm competition disadvantage has been very scarce. Using artificial insemination of eight +/+ females with equal sperm numbers from +/+ and +/*t* males, Olds-Clarke & Peitz (1985) inferred that the *t* haplotype was transmitted to 22% (5 out of 23) of the fetuses. This is a broad proxy of the +/*t* male's paternity share, because assignment depended on the tailless phenotype (genotype T/*t*) traditionally used for *t* haplotype detection. Consequently, paternity could be assigned only to offspring that inherited the *t* from their father and the tailless mutation T (brachyury) from their mother. Thus, accurate phenotypic paternity estimation relied on strong male drive, Mendelian inheritance of T in females and random fusion of the *t* and T gametes. Given these limitations and the small sample size associated with a large standard error, the authors were unable to conclude whether +/*t* paternity success was lower than expected from drive (the adjusted null hypothesis). Other studies suggesting a sperm competition disadvantage for +/*t* males based their estimate of paternity share on low numbers of multiply sired litters (Ardlie & Silver, 1996; Manser et al., 2011). Apart from being based on very few litters, these estimates are prone to a biased estimation of +/*t* male sperm competitiveness, as litters resulting from multiple mating but with exclusive paternity for one male would not have been included. In our mating trials, ejaculation by both males resulted in multiple paternity in only 17 out of 57 litters (29.8%) which is remarkably similar to estimates of multiple paternity from wild populations (Dean et al., 2006; Firman & Simmons, 2008b; Lindholm et al., 2013). If we had only analysed multiply sired litters, we would have overestimated +/*t* paternity share by a factor of almost three at 31.5%. Using controlled matings, we were able to overcome major limitations of previous studies and thus, to our knowledge, we

provide the first comprehensive estimate of +/*t* male disadvantage in postcopulatory competition.

1.5.2 +/t male ejaculate features

If sperm competition is the main explanation for the drastically reduced paternity share of +/*t* males, what sperm features might be causing this effect? While motile sperm from +/*t* males are hyperactivated sooner and show a faster initial rate of fertilization *in vitro*, their velocity and linearity are reduced (reviewed in Olds-Clarke, 1997). This results in a lower number of progressive sperm, reducing the number of sperm reaching the site of fertilization *in vivo* (Tessler & Olds-Clarke, 1981). These *t*-associated sperm motility features might relate to the paternity pattern found here. Our initial analysis suggested a first male benefit consistent with previous findings in mice (Firman & Simmons, 2008a). However, closer inspection revealed that differences in the number of ejaculations between competing males were responsible for this order effect (Figure 1.1). Thus, the absence of an order effect when accounting for the number of ejaculations was surprising. This suggests that +/*t* males ejaculate sperm that fail to benefit from the mating order typically favoured in this species (first male).

As an alternative to intrinsic sperm motility differences between +/+ and +/*t* males, sperm viability and motility of +/*t* males may be influenced by the seminal fluids of wild type males in sperm competition. In the stalk-eyed fly *Cyrtodiopsis whitei* that harbours a sex chromosome driver, the seminal fluid of wild type males incapacitates sperm from drive males, strongly reducing their fertilization success (Fry & Wilkinson, 2004).

1.5.3 Ejaculate allocation and female choice

Alternative explanations for the observed low paternity share other than intrinsic differences in sperm competitiveness between +/*t* and +/+ males are: (i) differential sperm investment depending on male genotype, and (ii) female choice.

(i) Males might employ different strategies for gaining paternity, such as differential investment into sperm production and differential ejaculate allocation. Here, the investigated organs involved in sperm production and sperm storage did not differ in size between +/+ and +/*t* males. This finding has to be interpreted with caution, as the intra-specific correlation between testis/epididymis weight and sperm production may be weak, and cryptic differences in testicular efficiency may remain undetected when looking at simple weight measurements (Firman et al., 2015a). However, in support of our findings, previous studies of congenic +/+ and +/*t* males consistently found no differences in the number of stored sperm (Olds-Clarke, 1997). The paternity outcome may also be attributed to differences in ejaculate allocation. Our finding that the number of ejaculations affects +/*t* male paternity share supports ejaculate allocation as a means by which males can affect the outcome of sperm competition. However, +/*t* males were not more likely to ejaculate twice than wild type males. In conclusion, given the strong effect of male genotype on paternity share and the significant effect of the number of ejaculations on paternity outcome, we deem it unlikely that comparably minor differences in sperm production or ejaculate investment are responsible for the low +/*t* paternity share in our experiment.

(ii) Females are known to discriminate between males and to show pre-copulatory mating preferences (Andersson, 1994). In a series of experiments testing olfactory and social female preference, +/*t* females preferred +/+ males over +/*t* males, while +/+ females showed no

preference (Lenington et al., 1992). A small paternity bias consistent with mate choice for genetic compatibility has also been found in a wild population (Lindholm et al., 2013). A recent study where females had free access to a +/*t* and +/+ male found paternity share to be lower for +/*t* than +/+ males, but was unable to distinguish between pre- and postcopulatory processes (Manser et al., 2015). Here, we measured the paternity outcome only when females received ejaculations by both males, thus the only avenue for female choice would be cryptic (Eberhard, 1996). In previous studies, transmission of the *t* haplotype was lower than expected in crosses in which +/*t* males were mated to +/*t* rather than +/+ females (Bateman, 1960; Lindholm et al., 2013), possibly indicating that females may be able to select genetically compatible sperm for fertilization. Although we cannot distinguish between sperm competition and cryptic female choice, we found no direct evidence for discrimination compatible with cryptic female choice for genetic compatibility, as female genotype did not affect the paternity outcome.

1.5.4 Fitness consequences for females

Owing to strong male drive and *t* homozygote lethality, +/*t* females mated monandrously to +/*t* males have much smaller litters than +/*t* females mated to +/+ males because many offspring from the former mating cross have the lethal genotype *t/t* (Carroll et al., 2004; Manser et al., 2015; this study). Here, we confirm that early embryo lethality in +/*t* females is a direct consequence of *t* homozygosity, as all detected *t/t* embryos were inviable. The proportion of viable embryos decreased with +/*t* male paternity share in +/*t* females but not in +/+ females. This has important implications for +/*t* females. By mating with more than one male, females can increase the probability of fertilization by a genetically compatible +/+ male. This appears to be a direct consequence of incompatible +/*t* males having a strong disadvantage in sperm competition. Lorch & Chao (2003) formally modelled selection for female multiple mating in the presence of fitness reducing mates. They concluded that multiple mating is only favoured when female fitness is a concave-down function of the proportion of costly mates, i.e., females mating with a costly and a non-costly male have less than half their offspring sired by the costly male (Lorch & Chao, 2003). We show that the female fitness function is indeed strongly concave-down (Figure 1.2) and thus that female multiple mating can be selectively favoured by the presence of the *t* haplotype. Compared to randomly mating monandrous females with an average +/*t* paternity share of 50%, polyandrous females reduce the +/*t* paternity share (to the left in Figure 1.2) with a positive effect on embryo viability (moving upwards in the top chart of Figure 1.2). When focusing on the immediate negative consequences as we did here, only +/*t* females benefit from polyandry. However, in natural populations, polyandrous +/+ females could also benefit from avoiding fertilization by +/*t* males by avoiding maternal investment into sons that are bad sperm competitors (Keller & Reeve, 1995). Similarly, females that invest into +/*t* daughters that face a risk of reduced reproductive success through genetic incompatibility are likely to have lower long-term fitness. Thus, all females potentially benefit from avoiding +/*t* males, but the magnitude of this benefit will depend on the genotype-specific benefits and the cost of polyandry (Manser et al., 2015).

1.5.5 Polyandry and the t *frequency paradox*

The *t* frequency in natural populations is typically dramatically lower than predicted by theory (the *t* frequency paradox; for a review, see Ardlie, 1998). As polyandry rates in natural house mouse populations are considerable (Dean et al., 2006; Firman & Simmons, 2008b; Lindholm et al., 2013), and females

show high remating rates in the laboratory (Rolland et al., 2003), our results strongly suggest that polyandry is likely to answer this long-standing puzzle in evolutionary genetics. Using a high rate of polyandry and a low sperm competitiveness of +/*t* males, a modelling approach showed that polyandry alone could account for the *t* frequency decline observed in the wild population from which our mice were derived (Manser et al., 2011). Polyandry might positively correlate with population density in wild populations, because females have more mating opportunities (Dean et al., 2006), which may account for the fact that *t* frequencies are typically much lower in large than in small populations (Ardlie, 1998).

1.5.6 Selfish genetic elements and polyandry

We found that an autosomal SGE has a strong impact on sperm competitiveness in house mice. Our results suggest that not only can polyandry prevent the spread of autosomal drive, but that polyandry is so effective at preventing fertilization by SGE bearing sperm, that even moderate costs to females associated with a driver could cause the evolution of increased polyandry. In modelling scenarios for sex-chromosome-linked male drive, Holman *et al.* (2015) found that polyandry can evolve as an effective response to sperm competition disadvantaged drive if there are additional costs to drive homozygotes.

Our results are in agreement with findings in other species bearing SGEs driving through males. Sex chromosome drivers in several plant and invertebrate species are associated with reduced competitive ability of male gametes, with 20–40% paternity share when averaged across mating order (Wu, 1983; Taylor et al., 1999; Wilkinson & Fry, 2001; Atlan et al., 2004; Wilkinson et al., 2006; Price et al., 2008a). Similar disadvantages in sperm competition have been found in studies investigating B chromosomes and cytoplasmic incompatibility inducing *Wolbachia* (Beukeboom, 1994; Champion de Crespigny & Wedell, 2006; Champion de Crespigny et al., 2008). Moreover, in response to a sex ratio biasing SGE, *Drosophila pseudoobscura* populations evolved higher remating rates and shorter remating latency after only 10 generations of experimental evolution (Price et al., 2008b). Here, we show that autosomal drive is associated with an extreme disadvantage in sperm competition in a mammal. Thus, our findings generalize the notion that male drivers cause a disadvantage in sperm competition (Price & Wedell, 2008).

Undetected autosomal drive that manipulates spermatogenesis could be common and is likely to incur fitness costs (Taylor & Ingvarsson, 2003; Burt & Trivers, 2006). If fitness costs of SGEs arise solely from genetic incompatibility, polyandry is not predicted to evolve even if SGE males have reduced sperm competitiveness (Champion de Crespigny et al., 2008). This is because the frequency at which females encounter incompatible mates determines the benefit of polyandry, which cannot offset even mild costs of polyandry when SGE carriers are rare (Champion de Crespigny et al., 2008; Wedell, 2013). However, if male carriers of SGEs are costly to all females, e.g., owing to reduced fertility, polyandry can readily evolve if SGE-carrying males are disadvantaged in sperm competition (Lorch & Chao, 2003; Wedell, 2013). Thus, it is possible that polyandry may have evolved, or may persist, in a wide range of species due to its benefits in resisting SGEs.

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Data are archived in the Dryad data repository: http://dx.doi.org/10.5061/dryad.m2f45.

CHAPTER 2 MEIOTIC DRIVE CHANGES SPERM PRECEDENCE PATTERNS IN HOUSE MICE: POTENTIAL FOR MALE ALTERNATIVE MATING TACTICS?

2.1 ABSTRACT

Background – With female multiple mating (polyandry), male-male competition extends to after copulation (sperm competition). Males respond to this selective pressure through physiological, morphological and behavioural adaptations. Sperm competitiveness is commonly decreased in heterozygote carriers of male meiotic drivers, selfish genetic elements that manipulate the production of gametes in males. This might give carriers an evolutionary incentive to reduce the risk of sperm competition. Here, we explore this possibility in house mice. Natural populations frequently harbour a well-characterised male driver (*t* haplotype), which is transmitted to 90% of heterozygous (+/*t*) males' offspring. Previous research demonstrated strong detrimental effects on sperm competitiveness, and suggested that +/*t* males are particularly disadvantaged against wild type males when first-to-mate. Low paternity success in the first-to-mate role is expected to favour male adaptations that decrease the risk of sperm competition by preventing female remating. Genotype-specific paternity patterns (sperm precedence) could lead to genetically determined alternative reproductive tactics that can spread through gene level selection. Here, we seek confirmation that +/*t* males are generally disadvantaged when first-to-mate and address whether males of different genotypes differ in reproductive tactics (copulatory and morphological) to maximise individual or driver fitness. Finally, we attempt to explain the mechanistic basis for alternative sperm precedence patterns in this species.

Results – We confirmed that +/*t* males are weak sperm competitors when first to mate. When two +/*t* males competed, the second-to-mate was more successful, which contrasts with first male sperm precedence when wild type males competed. However, we found no differences between male genotypes in reproductive behaviour or morphology that were consistent with alternative reproductive tactics.

Sperm of +/+ and +/*t* males differed with respect to *in vitro* sperm features. Premature hypermotility in +/*t* males' sperm can potentially explain why +/*t* males are very weak sperm competitors when first-to-mate.

Conclusions – Our results demonstrate that meiotic drivers can have strong effects on sperm precedence patterns, and may provide a heritable basis for alternative reproductive tactics motivated by reduced sperm competitiveness. We discuss how experimental and evolutionary constraints may help explain why male genotypes did not show the predicted differences.

Key words: t haplotype, sperm competition, selfish genetic element, multi-level selection, alternative reproductive tactics, polyandry, copulatory behaviour, ovulation, CASA

2.2 Background

Females of many species mate with multiple males (polyandry), leading to postcopulatory competition between males (Parker, 1970). With polyandry, a male's reproductive success is not only determined by his access to mates, but also by how successful his sperm are in competition for fertilisations (Møller, 1998). Males are predicted to respond to postcopulatory sexual selection through adaptations in ejaculate production and allocation (Simmons, 2001; Wedell et al., 2002). Alternatively, males may also attempt to reduce the risk of sperm competition by guarding females (Parker, 1970). The pay-off structure of different male tactics will depend on a number of factors (Parker, 1974; Alcock, 1994). One of the most important determinants of the pay-off to mate guarding is sperm precedence, the distribution of paternity share among males in sperm competition (Parker, 1974; Alcock, 1994; Alonzo & Warner, 2000; Harts & Kokko, 2013). With last male sperm precedence, i.e., where the last-to-mate male sires the majority of offspring, the potential fitness loss to a first-to-mate male due to sperm competition is larger than with first male precedence. As a consequence, more investment into mate guarding is predicted with last male precedence (Parker, 1974).

Intrinsic variation between males can cause variation in male alternative reproductive tactics (ART). Male ARTs define different ways of intraspecific and intrasexual competition for paternity (Taborsky et al., 2008) and typically involve a set of correlated behavioural, physiological and/or morphological traits. The main factors thought to lead to ARTs are differences in the ability to defend females or resources (Taborsky et al., 2008). In fish, large males often follow a bourgeois tactic including mate guarding and parental care, whereas small males with relatively large testes usually follow a parasitic tactic with sneak fertilisations (Taborsky, 2008). The possibility that intrinsic variation in sperm competitiveness can cause variation in male reproductive tactics has received little attention. However, Engqvist (2012)] modelled optimal ejaculate allocation for males that intrinsically vary in sperm competitiveness as a consequence of mitochondrial variation or segregation distorters that act in males. His findings highlight the potential for ARTs as a consequence of intrinsic male variation, with differential allocation between male types especially when intrinsic differences between males are pronounced and polyandry levels are moderate (Engqvist, 2012).

A potentially wide-spread origin of variation in postcopulatory competitiveness is segregation distortion in males (Price & Wedell, 2008). Meiotic drivers are selfish genetic elements that interfere with fair Mendelian segregation in diploid organisms, and as a consequence are inherited by more than 50 % of the offspring (hence they 'drive'; Burt & Trivers, 2006; Lindholm et al., 2016). If meiotic drive elements cannot reach fixation, for example due to homozygote lethality, a polymorphism at the drive locus can persist (Lindholm et al., 2016). Drive occurs in heterozygotes, and typically in males (Taylor & Ingvarsson, 2003), the driver kills or interferes with gametes not carrying the driver. As a consequence, male carriers have fewer viable or functional sperm, with important negative consequences for their sperm competitiveness (Haig & Bergstrom, 1995; Zeh & Zeh, 1996). Empirical evidence supports the notion that male drive commonly reduces sperm competitiveness (Price & Wedell, 2008; Wedell, 2013). Drive elements thus provide a heritable genetic basis for sperm competitiveness, with potential implications for male ARTs. Especially interesting is that fitness of drive-carriers does not have to exceed that of non-carriers for drive-associated ARTs to spread,

as fitness accounting takes place at the gene level, and includes the transmission advantage from drive.

The *t* haplotype in house mice is a classic example of male drive that has a long evolutionary history of around 3 million years (Morita et al., 1992). Previous research shows that male carriers (denoted as $+/t$) are strongly disadvantaged in sperm competition against wild type (+/+) males (Chapter 1). Drive in $+/t$ males is due to an elaborate molecular mechanism comparable to a "poison-antidote" system that results in abnormal flagellar function of + sperm within a $+/t$ ejaculate (Herrmann & Bauer, 2012). At least four distorters (the "poison") and the responder (the "antidote") are part of the *t* haplotype's large set of linked genes that are protected from recombination by four major inversions that take up about one third of chromosome 17 (Herrmann & Bauer, 2012). Gamete interference within the $+/t$ ejaculate results in the majority (typically around 90 %) of the offspring of a $+/t$ male inheriting the *t*, while transmission follows the fair rules of mendelian inheritance in female carriers. An important aspect of the sperm competition findings is that $+/t$ males obtain a very small paternity success when competing against wild type males, indicating that the "poison-antidote" system of the *t* haplotype leaves *t* sperm partially impaired (Chapter 1). Curiously, there is no order effect in $+/t$ versus +/+ sperm competition, contrasting with the first male sperm precedence previously described for house mice (Levine, 1967; Firman & Simmons, 2008a), and suggesting that $+/t$ males may not be able to benefit from the usual first-to-mate advantage (Chapter 1). Given their very weak sperm competitiveness, $+/t$ males might follow a reproductive tactic where they attempt to secure paternity by preventing sperm competition. Female house mice have been shown to be actively polyandrous in the lab (Rolland et al., 2003; Manser et al., 2015), and multiple paternity is common in wild-caught females (Dean et al., 2006; Firman & Simmons, 2008b; Auclair et al., 2014). A strong disadvantage particularly in the defensive (i.e., first-to-mate) sperm competition role should strengthen $+/t$ males' incentive to prevent female remating with other males. Depending on the efficacy of prevention of female remating, an increased effort by $+/t$ males could compensate for the disadvantage and result in equal fitness for both genotypes, or alternatively, $+/t$ males could be doing the "best of a bad job" (Lee, 2005). Interestingly, the same argument can be applied at the gene level, where fitness might be the same for the *t* haplotype and its wild type counterpart, or the *t* haplotype could be doing the best of a bad job. Differences in behaviour between $+/t$ and +/+ mice in other contexts demonstrate the *t* haplotype's potential to influence behavioural traits, although there is limited consistency across studies: genotypes may differ with respect to female preference (Lenington, 1991; but see Manser et al., 2015), male social dominance ($+/t > +/+$ (Lenington et al., 1996) and $+/t < +/+$ (Carroll et al., 2004)), and female personality and life-history strategy (Auclair et al., 2013).

Here, we investigate in house mice whether $+/t$ males are indeed generally disadvantaged in defensive sperm competition and how that might affect male reproductive tactics. The pay-offs of alternative tactics can strongly depend on sperm precedence patterns (Parker, 1974). First, we compare the paternity outcome from sperm competition between two $+/t$ males to sperm competition between two +/+ males. We then explore the possibility of alternative reproductive tactics in males by measuring a suite of behavioural and morphological traits related to reproduction. Male house mice might have a variety of possibilities to influence the risk of sperm competition. Later ejaculation relative to oestrus stage may benefit $+/t$ males when they are first to mate by reducing the time available for (and thus the likelihood of) female remating (Preston & Stockley, 2006). Similarly, extended copulatory stimulation may reduce sperm competition risk by reducing female receptivity to other males (Huck & Lisk, 1986; Dewsbury, 1988b; Stockley & Preston, 2004),

and repeated ejaculation provides a paternity advantage in mice (CHAPTERS 1 & 5). Large copulatory plugs produced by proteins from the seminal vesicle and coagulating gland can delay female remating and increase paternity share of first-to-mate males (CHAPTER 5). Plugs thus offer some potential to increase reproductive success through passive mate guarding (DUNHAM & RUDOLF, 2009; FROMHAGE, 2012). Alternatively, investing into scent marking to signal social dominance and territory ownership (HURST & BEYNON, 2004) and to attract females (ROBERTS & GOSLING, 2003) via proteins from the preputial gland may increase reproductive success (THONHAUSER ET AL., 2013A). We address the possibility of alternative reproductive tactics in +/*t* and +/+ males by observing copulatory behaviour and assessing investment into different male reproductive organs that account for the production of ejaculate components and scent marks. Finally, we attempt to mechanistically link the sperm precedence patterns to sperm phenotypes by assessing temporal dynamics of sperm features *in vitro*.

2.3 MATERIAL AND METHODS

2.3.1 Experimental animals

Study subjects were male and female wild house mice (*Mus musculus domesticus*) that were laboratory-born F1 to F3 descendants from a free-living population near Illnau, Switzerland (KÖNIG & LINDHOLM, 2012), from which we introduce wild-caught individuals into our breeding colony every generation. We bred and kept mice under standard laboratory conditions under a 14L:10D cycle (breeding colony: lights on at 05:30 CET; mating experiments: reversed cycle with lights on at 17:30 CET) at a temperature of 22-24 °C with food (laboratory animal diet for mice and rats, no. 3430, Kliba) and water provided *ad libitum*, and paper towels and cardboard served as enrichment and nest building material. Our laboratory population is derived from a wild population that harboured a single *t* haplotype variant with strong male drive and homozygote lethality (LINDHOLM ET AL., 2013). Breeding pairs consisted of monogamous pairs of non-sibling +/+ males and either +/+ or +/*t* females, the latter producing on average 50% +/*t* offspring. At the age of 23 days, we weaned offspring, took a tissue sample by ear punch for genotyping and individual identification, and kept them in same sex sibling groups in Makrolon Type III cages (23.5 x 39 x 15 cm). We used +/*t* and +/+ males and females and diagnosed their *t* haplotype status before they entered the experiment. DNA extraction was performed by salt-chloroform extraction (MÜLLENBACH ET AL., 1989) and *t* haplotype status was diagnosed as described elsewhere (SCHIMENTI & HAMMER, 1990; LINDHOLM ET AL., 2013). Male mice were separated at latest at the onset of aggression between brothers and kept individually in Makrolon Type II cages (18 x 24 x 14 cm). Mice were moved from the breeding colony room into the experimental at least two weeks before being used in the experiment to allow for acclimatisation to the reversed light cycle. The experimenter was blind with respect to genotype during all procedures, including mating trials, female and male dissections, sperm analyses, and video observations (see below).

2.3.2 Sperm competition trials

For this study, we made use of sperm competition trials from an experiment on the effect of copulatory plugs on rival (second-to-mate) male behaviour and paternity outcome (CHAPTER 5). We used both +/*t* and +/+ males and females, focusing on competition between brothers of the same

t genotype for paternity data. Two full brothers from the same litter competed against each other in order to control for potential effects of genetic background and maternal environment on sperm competitiveness. For behavioural analyses, we focus on first-to-mate males and how their copulatory behaviour may relate to reducing the risk of sperm competition. Mating trials were conducted as specified elsewhere (CHAPTER 5). Briefly, a sexually receptive female (BYERS ET AL., 2012) was introduced into a male's cage. Trials were started 2.5 h ± 0.5 (mean ± SD) after the beginning of the dark phase, and females were subsequently checked for a copulatory plug (indicating ejaculation; MCGILL, 1962) every 1–1.5 h. Once a plug was detected, the trial was stopped and the plug was either removed or left intact (CHAPTER 5), after which the female was paired with the second male and checked every 30–60 min until either a new copulatory plug was observed or until the beginning of the next dark phase. After the second mating, plugs were again removed or left intact, and mated females were kept in isolation. Females that did not mate were re-tested on a later occasion. We used a paired design for our plug removal treatment (CHAPTER 5), so that males were used in multiple mating trials.

Paternity assignment – To get paternity estimates that were unbiased by embryonic mortality associated with homozygous effects of the *t* haplotype (SAFRONOVA, 2009; CHAPTER 1), we sacrificed females 9 days *post coitum* using gradual CO_2 filling in their home cage and recovered embryos under a dissection microscope at 10–40x magnification. Paternity was assigned using the software CERVUS (KALINOWSKI ET AL., 2007) on genotypes from 12 microsatellites spread across 10 autosomes, with details as described elsewhere (CHAPTER 1).

2.3.3 Alternative reproductive tactics?

Copulatory behaviour – We used video recordings to obtain detailed information on copulatory behaviour of both males. The first male's first ejaculation would sometimes go undetected during a trial, when the male dislodged his own plug after ejaculation but before the female was checked for the presence of a plug. For paternity analyses, we recorded the number and timing of both males' ejaculations, as reported previously (CHAPTER 5). Here, we additionally recorded details on first-to-mate males. From the first copulatory series, we recorded (i) the latency from introduction to the first mount, (ii) the number and (iii) average duration of copulatory bouts (mounts and mounts with intromission), (iv) the latency from the first copulatory mount to ejaculation, and (v) the *in copula* duration at ejaculation. As a proxy for the male's motivation to repeatedly mate with the same female, we also assessed (vi) the latency from ejaculation to the initiation of a second copulatory series (post-ejaculation interval).

Ejaculation timing relative to ovulation – To investigate potential differences between male genotypes in their ejaculation timing relative to ovulation, we used the extent of cornification of epithelial cells in vaginal smears as a proxy for female oestrus stage (PRESTON & STOCKLEY, 2006; BYERS ET AL., 2012). We took vaginal smears using plastic inoculation loops and took digital photographs under a microscope at 100x magnification. Images were scored by a single observer for the proportion of cornified epithelial cells at steps of 0.1. Oestrous stage scores from 50 pictures assessed independently on two different days showed high intra-observer repeatability for scoring ($F_{49,50} = 13.1$, $p < 0.001$, $R = 0.928$).

Male reproductive organs – Here, we investigated whether weak sperm competitors (+/*t* males) invest differently into traits important for pre- versus postcopulatory selection than strong sperm competitors (+/+ males). For all males involved in mating experiments and sacrificed for sperm analyses (see below), we measured the relative organ weights of the

preputial glands (pheromone production), testes and the entire epididymides (sperm production and storage), and seminal vesicles and coagulating glands (copulatory plug production; dissected pairwise).

2.3.4 Sperm features

To investigate effects of the *t* haplotype on sperm features, we compared sperm features of +/+ and +/*t* males *in vitro*. Full details of the procedures are provided as supplementary methods (SUPPLEMENT B). Briefly, we analysed sperm of 12 pairs of sexually mature +/*t* and +/+ brothers from monogamous breeding pairs. Males were kept in isolation and were sacrificed using gradual CO_2 filling in their home cage. The order of dissection was randomised and all procedures were done blind. We dissected both caudal epididymides and incubated sperm in modified human tubal fluid (mHTF; Bühlmann Laboratories AG) at 37 °C. Using computer assisted sperm analysis (CASA; MouseTraxx, Hamilton Thorne), we measured patterns of sperm velocity and linearity (average path velocity VAP, straight-line velocity VSL, curvilinear velocity VCL, amplitude of lateral head displacement ALH, beat cross frequency BCF, straightness STR, and linearity LIN). Repeated measurements over a large time span have been recommended for obtaining data on both initial swimming speed and the rate of decline (REINHARDT & OTTI, 2012). We attempted to cover the time period that sperm are stored *in vivo* between ejaculation and ovulation, which has previously been estimated at between 2 and 5 h in a monogamous context in laboratory mice (SNELL & FEKETE, 1940; BRADEN, 1957). Thus, for every male we measured a large number of sperm paths (mean $\pm$ SD = 327 $\pm$ 270) at each of 4–5 time points after different incubation times between 15 min and 6 h.

2.3.5 Statistical analyses

An overview of the sample sizes available for the different analyses is given in Table 2.1 The data set supporting the results of this article is available in the Dryad repository, doi:10.5061/dryad.m2h55. Using the functions lmer and glmer in lme4 (BATES ET AL., 2014) in R version 3.1.3 (R CORE TEAM, 2015), we analysed data on paternity outcome, sperm features, copulatory behaviour and reproductive organs with either linear mixed models (LMM), or generalised mixed models (GLMM). We extracted effect sizes from full models to avoid biasing effect sizes through removal of non-significant terms (FORSTMEIER & SCHIELZETH, 2011). To test the global null hypothesis, we compared full models to null models using likelihood ratio tests (FORSTMEIER & SCHIELZETH, 2011). For LMMs, we obtained p-values for fixed effects using F-tests between full models and a model excluding the factor of interest, with degrees of freedom based on the Kenward-Roger approximation implemented in the package pbkrtest (HALEKOH & HØJSGAARD, 2014). To improve interpretability, some continuous input variables were standardised to a mean of 0 and a standard deviation of 1 (see Table 2.2) as recommended by Schielzeth (2010). We calculated approximate 95 % confidence intervals (c.i.) by multiplying Student's *t*-values for our sample sizes by standard errors of the predicted values (CRAWLEY, 2007).

P_2 – We analysed the proportion of embryos sired by the second male (P_2) with binomial GLMMs. The number of embryos sired by the second male was included as the dependent variable and the number of offspring genotyped as the binomial denominator. To investigate sperm precedence patterns in relation to the genotype combination of competing males, we ran a GLMM on P_2, with the full model including the following variables: male genotype combination (factor

with two levels), the body weight difference of the two males, the difference in ejaculation numbers of the males, the interval between both males' first ejaculations, and female genotype. Male identity was included as a random effect to avoid pseudoreplication. Dispersion parameters of the GLMMs were ≈ 1.

Table 2.1

Subsection	Sample sizes	Male genotype combination			
		+/*t* vs +/*t*	+/*t* vs +/+	+/+ vs +/*t*	+/+ vs +/+
Sperm precedence	N mating trials (N embryos)	17 (117)	–	–	23 (179)
Copulatory behaviour	N mating trials (N different individual males)	17 (10)	10 (4)	9 (4)	39 (14)
		Male genotype			
		+/*t*		+/+	
Male reproductive organs	N males	40		48	
Sperm features	N males	12		12	

Overview of sample sizes available for the different analyses.

Copulatory behaviour – Our recorded variables were not sufficiently correlated to justify a reduction of dimensionality. Thus, we analysed the components of copulatory behaviour individually using LMMs. Full models contained male and female body weight, and male and female genotype as fixed effects. To investigate whether males adjust the timing of their ejaculation to female oestrus stage, we included the proportion of cornified cells (our measure of oestrus stage) and its interaction with male genotype as additional covariables. We included male identity as a random effect to avoid pseudoreplication. Full models were compared to null (intercept-only) models using likelihood ratio tests on the global null hypothesis that the focal behaviour was unaffected by any of the included fixed effects (Forstmeier & Schielzeth, 2011).

Because post-ejaculation interval included many (30/83) right-censored data points (when trials were discontinued after detection of a plug and the male had not yet performed any post-ejaculatory mounts), we analysed it with a cox proportional hazard model in the survival package (Therneau, 2015).

Male reproductive organs – The weights of preputial glands, testes, epididymides, and seminal vesicles and coagulating glands were analysed using LMMs with brother pair as a random effect to account for similarity caused by relatedness and shared early environment. As fixed effects we included male body weight, male genotype and their interaction term.

Sperm features – We measured sperm traits from 25,284 individual sperm in 828 scans at 4–5 different time points for each for 24 males. Mean values per sperm sample may be a poor representation of a sample's fertilisation potential or competitiveness, given that most sperm will not make it to the fertilisation site (Reinhardt & Otti, 2012). In the context of the *t* haplotype, the drive mechanism reduces the fertilisation potential of a large proportion of a +/*t* male's sperm. Moreover, in our *in vitro* measurements, a considerable proportion of the measured sperm stuck to the cover slide (see Li et al. (2015) for how to avoid this problem). Indeed, many of our sperm

variables showed a bimodal distribution, most likely as a result of having both stuck and free swimming sperm in our samples. For all these reasons, we subset our dataset to include only the upper 50 % per sample, based on curvilinear velocity (since this velocity measure is least affected by the shape of sperm movement). Sperm traits were correlated and were reduced using principal components analysis (PCA) using the function *principal* in the psych package (Revelle, 2015). Both Bartlett's and Steiger's tests clearly rejected the null hypothesis that all correlations between traits were zero (see Table S4), the Kaiser-Meyer-Olkin measure of sampling adequacy was moderate at 0.57 (calling for a cautious interpretation), and parallel analysis suggested that extracting two components was adequate. Components were rotated using the orthogonal varimax method and scores were calculated using regression. We then averaged scores to obtain a single value for a given male at a given incubation time ($N = 104$) for each component, and the two components were then analysed using LMMs. The full models contained the male's genotype, incubation time, the number of sperm counted (averaged across replicate scans; range = 20–176, corresponding to 1.3–12.8 million sperm/mL) and all two-way interactions as fixed effects. Since we had repeated measures and a sibling design, we included individual-specific random intercepts (nested within male brother pair) and individual-specific random slopes for incubation time to avoid overconfidence in interaction estimates (Schielzeth & Forstmeier, 2009). The percentage of motile sperm was averaged across replicate scans and was analysed separately.

2.4 Results

2.4.1 Sperm precedence

In a controlled sperm competition experiment, we mated female house mice consecutively to two different males. We analysed paternity data from sperm competition trials in which two $+/t$ males or two $+/+$ males had competed, and successfully assigned paternity for 311 of the 332 embryos dissected from 42 pregnant females. The paternity share of the second male to mate (P_2) ranged from zero to one, with many incidences of exclusive paternity for one of the males (48 %) despite multiple mating. P_2 varied strongly with the combination of male genotypes. Mean P_2 was 0.27 when two $+/+$ males competed, but rose to 0.72 when two $+/t$ brothers competed (raw data in Figure 2.1). We then investigated in more detail which factors determined paternity success, incorporating behavioural data on timing and number of ejaculations. A full model on 40 trials with complete information (Table 2.1; $n = 23$ for $+/+$ vs $+/+$; $n = 17$ for $+/t$ vs $+/t$) showed significant effects of the *t* haplotype (versus wild type), the difference in the number of ejaculations, the interval between the two males' ejaculations, and female genotype (Table 2.2). Repeated ejaculation by the first male decreased P_2 ($z = -2.31$, $p = 0.038$), as did a longer delay between the first and the second males' ejaculation ($z = -3.88$, $p < 0.001$). P_2 was higher in $+/t$ females ($z = 1.96$, $p = 0.048$). After controlling for other factors, mean P_2 was predicted at 0.15 [95 % c.i. = 0.06, 0.31] for competition between two $+/+$ males and 0.93 [0.76, 0.98] for competition between two $+/t$ males, respectively (Figure 2.2, model predictions). Thus, both P_2 predictions were highly significantly different from equal paternity share, but showed an inversion from first male sperm precedence when two $+/+$ males competed to second male precedence when two $+/t$ males competed. We combined paternity success data in a payoff matrix to compare individual level and gene level success, taking into account the transmission advantage of the *t* haplotype (Table 2.3). Comparing the relative fitness at the gene level, the *t*

haplotype has a maximum of 0.23 of the fitness of its wild type counterpart in sperm competition when first-to-mate but 1.88 when second-to-mate, and 1.8 without sperm competition. Rival male genotype does not strongly influence these pay-offs.

2.4.2 Alternative reproductive tactics?

Copulatory behaviour – From our sperm competition trials, we recorded detailed copulatory behaviour for 83 first-to-mate males. Figure 2.2 shows variation in copulatory behaviour in relation to male genotype at the *t* locus. Summary statistics for copulatory behaviour of +/*t* and +/+ males are given in Table S2. Comparisons of full models for the different aspects of copulatory behaviour to their respective null models revealed that the global null hypotheses could not be rejected (Table S2). Thus, neither mount latency, the number or average duration of copulatory bouts (log transformed), latency to ejaculation (sqrt transformed) and *in copula* duration at ejaculation showed any strong evidence for an association with male or female genotype or body weight, or with oestrus stage (Full model tests: all $p > 0.08$; $N = 75$ trials with complete information). The only association between behaviour and a phenotypic or genotypic variable was that heavier males had a shorter ejaculation latency ($F_{1,37} = 5.20$, $p = 0.028$), though this became non-significant when accounting for multiple testing ($p = 0.096$). Univariate analyses on the effect of male genotype did not support any influence of the *t* haplotype on copulatory behaviour (Table S2; all $P > 0.254$; $n = 83$).

In addition, we used data on post-ejaculation interval from 53 trials (19 of which involved +/*t* males), complemented with right-censored data from 30 trials (13 trials with +/*t* males) to ask whether +/*t* and +/+ males showed different behaviour. The cox proportional hazard model met the proportional hazards assumptions and indicated no difference between +/*t* and +/+ males ($\exp(\beta) = 1.18$, $p = 0.573$).

Male reproductive organs – There was no difference between the body weight of +/*t* and +/+ males (mean ± SD: +/*t* males 26.3 ± 2.0 g; +/+ males 26.5 ± 2.4 g; $F_{1,71} = 0.73$, $p = 0.396$). The weights of preputial glands (log transformed), testes and epididymides were correlated with body weight (preputial: $F_{1,69} = 27.74$, $p < 0.001$; testes: $F_{1,82} = 14.46$, $p < 0.001$; epididymides: $F_{1,78} = 32.40$, $p < 0.001$) but showed no significant differences between +/*t* and +/+ males (preputial: $F_{1,85} = 1.06$, $p = 0.307$; testes: $F_{1,79} = 0.71$, $p = 0.403$; epididymides: $F_{1,82} = 3.08$, $p = 0.083$). Seminal vesicles and coagulating glands (weighed pairwise) also correlated positively with body weight ($F_{1,77} = 35.22$, $p < 0.001$) and showed a significant difference between +/*t* and +/+ males ($F_{1,68} = 5.27$, $p = 0.025$). Thus, +/+ males had heavier seminal vesicles and coagulating glands relative to body weight than +/*t* males (predicted mean difference [95 % c.i.] = 11.6 mg [2.0, 21.2] = 6 % [1 %, 11 %] of the total weight). The interaction between male body weight and genotype was not significant for any of the organs (all $p > 0.161$).

2.4.3 Sperm features

We obtained measurements of features of sperm that had left the epididymis during 10 min initial incubation. We repeatedly measured these samples 4–5 times each over several hours of *in vitro* incubation for 24 males (12 +/+ and +/*t* full brothers). Summary statistics of sperm features for +/+ and +/*t* males for different time periods are given in Table S5. We analysed sperm features from a PCA on 121 ± 93 sperm from each of 4–5 time points for 24 males. The two extracted principal components are summarised in Table S3 and the correlation matrix is given in Table S4. The first component (PC1) explained 49.5 % of the variation in sperm features and was positively

loaded by measures of path straightness and linearity (STR, LIN) and the smoothed and linear speed (VAP, VSL). The second component (PC2) explained 26.9 % of the variation and was positively loaded by the speed and displacement of the sperm head (VCL, ALH) and by the smoothed path velocity (VAP). Thus, males with higher PC1 scores had linear and progressive sperm, whereas males with higher PC2 scores had sperm whose heads moved vigorously. A combination of low PC1 values and high PC2 values is an indication for hypermotility (see Discussion), vigorous nonlinear movement triggered during activation of mammalian sperm.

Full model results for both components are shown in Table 2.2 and illustrated in Figure 2.3. Progressive sperm speed (PC1) was lower for $+/t$ than for $+/+$ males (main effect b [95 % c.i.] $=-0.35$ $[-0.66,-0.04]$ for an incubation time of zero and centred for sperm numbers; $F_{1,19} = 4.44$, $p = 0.048$). Progressive speed also decreased over time and with higher sperm density, but there were no significant interactions (all $p > 0.5$). Sperm head speed (PC2) decreased over time, but tended to do so faster for $+/t$ than for $+/+$ males (interaction genotype x incubation time -0.06 $[-0.11, 0.003]$; $F_{1,19} = 3.07$, $p = 0.096$). Sperm count did not have any significant effect on PC2.

Sperm count did not change over time ($F_{1,21} = 0.02$, $p = 0.877$), but tended to be higher for $+/t$ males ($F_{1,11} = 3.47$, $p = 0.088$). The percentage of motile sperm tended to be initially higher for $+/t$ than for $+/+$ males ($F_{1,11} = 4.52$, $p = 0.056$). Additionally, there was a trend for an interaction between male genotype and incubation time ($F_{1,21} = 3.44$, $p = 0.078$). Thus, the higher percentage of motile sperm of $+/t$ males tended to increase over time, whereas for $+/+$ males it decreased slightly.

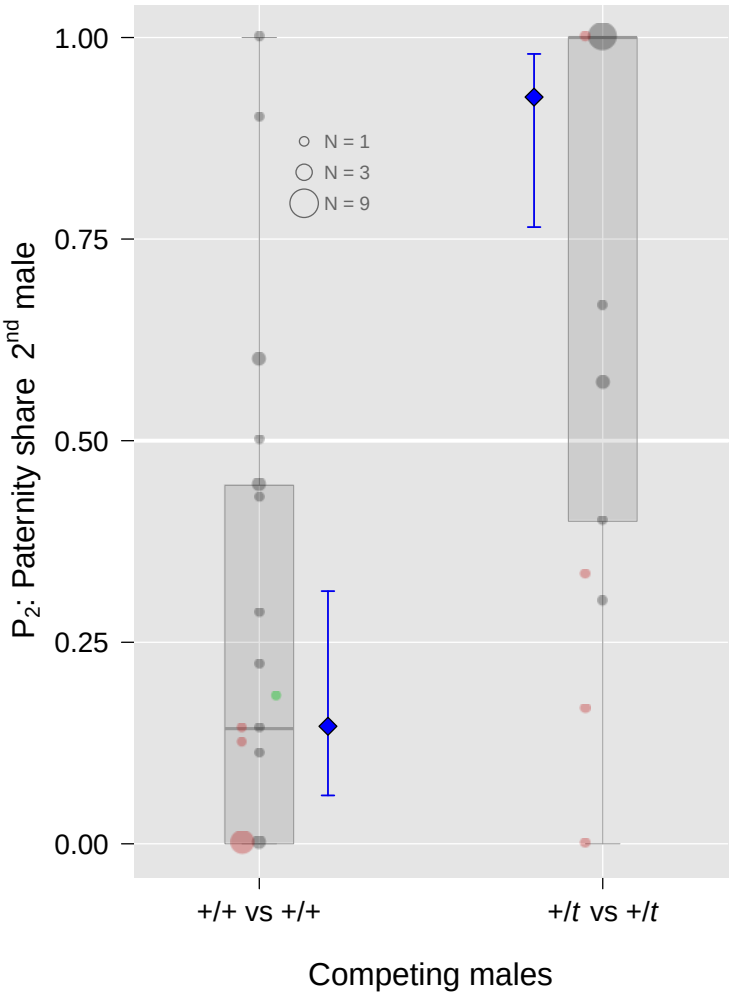

Figure 2.1

Sperm precedence patterns change with male genotype combination. Shown is the paternity share of the second-to-mate male (P2). P2 was below 0.5 (first male precedence) when two +/+ males competed, but above (second male precedence) when two +/*t* males competed. Boxplots and circles show the raw data, with area size corresponding to the number of observations. Red circles depict sperm competition trials in which the first male ejaculated twice; the green circle shows a trial with two ejaculations by the second male. Blue diamonds and error bars show the model predictions and 95 % confidence intervals from a GLMM accounting for other fixed effects (see main text).

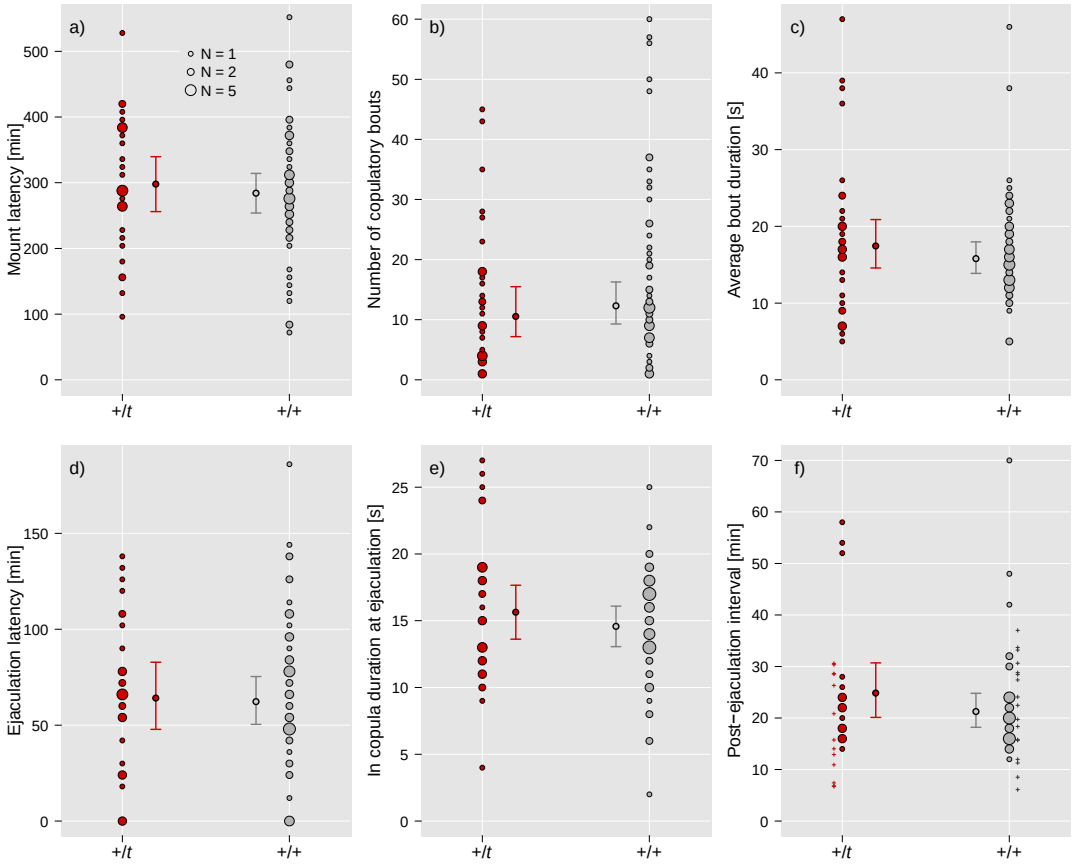

Figure 2.2

Copulatory behaviour of first-to-mate males. Shown is variation in six different aspects of copulatory behaviour of +/*t* and +/+ males: (a) mount latency, (b) the number of copulatory bouts, (c) average duration of copulatory bouts, (d) ejaculation latency, (e) in copula duration at ejaculation, and (f) post-ejaculation interval. Right-censored data for post-ejaculation interval are indicated with plus symbols (i.e., minimum times for males that were separated from the female before performing post-ejaculatory mounts; see main text). None of the behaviours showed a significant association with male genotype at the *t* locus (see main text and Table S3). +/*t* males are shown in red, +/+ males in grey. Points and error bars depict model predictions and approximate 95% confidence intervals obtained from full models (LMMs, back-transformed to the original scale where necessary).

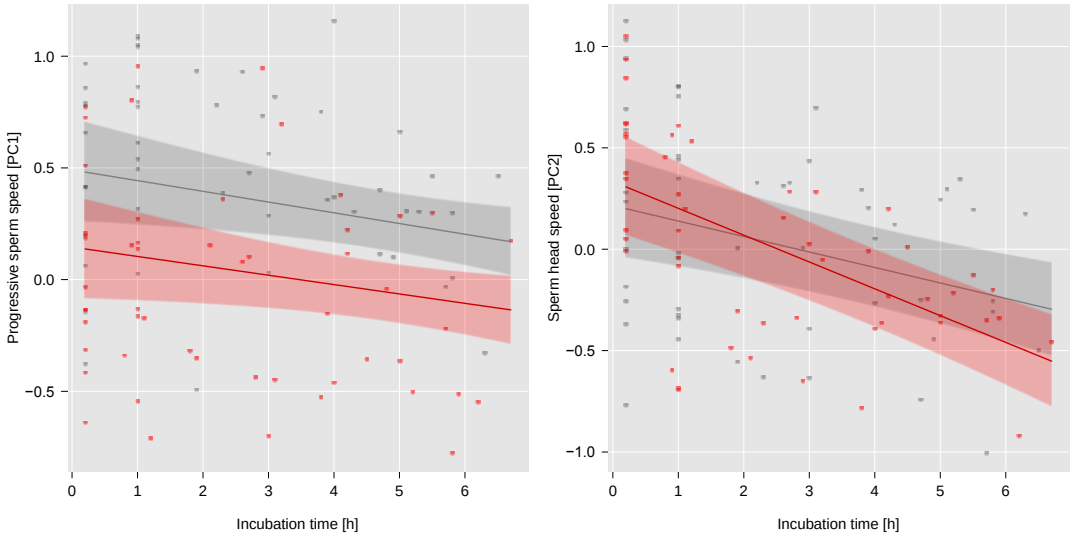

Figure 2.3

Temporal dynamics of *in vitro* progressive sperm speed (PC1; left panel), and sperm head speed (PC2; right panel). Raw data are shown as red (+/*t* males) and grey circles (+/+ males). Lines correspond to predictions from full models (random slope LMMs) including interaction terms and centred for sperm count. Shaded areas depict approximate 95% confidence intervals. The interaction between *t* haplotype genotype and incubation time was not significant for PC1 ($P = 0.850$) and tended to be negative for PC2 ($P = 0.096$; see main text and Table 2.2). Thus, sperm linearity and progressiveness (PC1) decreased over time for both male genotypes. In contrast, sperm head speed (PC2) tended to decrease more strongly for +/*t* than for +/+ males (right panel). For principal component loadings, see Table S3.

Table 2.2

Model	Response variable	Random effects	Fixed effects	Mean (SD)	Fixed effect centred/ standardised?	Estimate [approx. 95% CI]	*z*value/ F value	p
GLMM	P_2:	Male ID	Intercept (competition +/+ vs +/+)			**-1.47 [-2.39, -0.54]**	-3.21	**0.001**
	Paternity share 2nd male		Male genotype combination (+/*t*vs +/*t*)	–	n/n	**4.30 [2.55, 6.04]**	4.99	**< 0.001**
			Ejaculation number 1st male - 2nd male	0.3 (0.5)	n/n	**-1.39 [-2.75, -0.04]**	-2.08	**0.038**
			Ejaculation interval 1st to 2nd male [h]	2.0 (0.9)	y/y	**-1.39 [-2.12, -0.67]**	-3.88	**< 0.001**
			Weight difference 1st male - 2nd male	-1.3 (2.2)	y/y	0.22 [-0.42, 0.86]	0.70	0.484
			Female genotype (28 +/+; 12 +/t)	–	y/n	**1.33 [-0.03, 2.69]**	1.96	**0.048**
LMM	PC1:	Brother pair/Male ID	Intercept (centred for genotype)			**0.32 [0.15, 0.48]**	–	–
	Progressive sperm speed	Incubation time*Male ID	*t*haplotype	–	y/n	**-0.35 [-0.66, -0.04]**	4.44	**0.048**
			Incubation time [h]	2.5 (2.0)	n/n	**-0.05 [-0.07, -0.02]**	9.80	**0.005**
			Sperm count	71.3 (36.7)	y/y	**-0.28 [-0.38, -0.17]**	23.60	**< 0.001**
			*t*haplotype*Time	–	–	0.01 [-0.05, 0.06]	0.04	0.850
			*t*haplotype*Sperm count	–	–	0.05 [-0.08, 0.18]	0.43	0.517
			Sperm count*Time	–	–	0.01 [-0.02, 0.03]	0.40	0.528
LMM	PC2:	Brother pair/Male ID	Intercept (centred for genotype)			**0.27 [0.08, 0.47]**	–	–
	Sperm head speed	Incubation time*Male ID	*t*haplotype	–	y/n	0.12 [-0.18, 0.41]	0.53	0.476
			Incubation time [h]	2.5 (2.0)	n/n	**-0.10 [-0.13, -0.08]**	48.10	**< 0.001**
			Sperm count	71.3 (36.7)	y/y	-0.09 [-0.22, 0.03]	1.75	0.190
			*t*haplotype*Time	–	–	-0.06 [-0.11, 0.003]	3.07	0.096
			*t*haplotype*Sperm count	–	–	0.10 [-0.06, 0.27]	1.17	0.285
			Sperm count*Time	–	–	0.005 [-0.03, 0.04]	0.06	0.809

Full model summaries for P2 and sperm features. LMM linear mixed model, GLMM generalised linear mixed model. Intercepts correspond to estimates for wild type (+/+) males (paternity share) or were centred for male genotype (sperm features) by assigning values of -0.5 and +0.5 to +/+ and +/*t* males, respectively. Thus, intercepts in the sperm models correspond to an average between +/+ and +/*t* males for an incubation time of 0 h, and *t* haplotype shows the change in sperm features for +/*t* relative to +/+ males. Approximate 95 % confidence intervals were obtained by multiplying Student's t-values for our sample sizes by standard errors of the predicted values (Crawley, 2007). 95 % confidence intervals not overlapping zero and *P* values < 0.05 are highlighted in bold. Degrees of freedom for F values were based on the Kenward-Roger approximation (Halekoh & Højsgaard, 2014).

Table 2.3

	Monandry	Polyandry			
		First-to-mate		Second-to-mate	
	No rival	+/+ rival	+/*t* rival	+/+ rival	+/*t* rival
Paternity share focal +/+ male	1	0.85	0.89	0.15	0.89
Paternity share focal +/*t* male	1	0.11	0.07	0.11	0.93
Relative fitness +/*t* male	**1**	**0.13**	**0.08**	**0.73**	**1.04**
Relative fitness *t* haplotype	**1.8**	**0.23**	**0.14**	**1.32**	**1.88**

Pay-off matrix for +/+ and +/*t* males for different mating scenarios, and relative fitness for +/*t* males and the *t* haplotype. Paternity estimates are based on GLMM model predictions for a scenario where both males ejaculate once and the interval between the first and second male's ejaculation as well as female genotype are centred (see results). Paternity shares for sperm competition between +/+ and +/*t* males are taken from CHAPTER 1. Relative fitness (indicated in bold) is expressed for the +/*t* male (the *t* haplotype), with the fitness of the +/+ male (the *t* haplotype's + counterpart) set to one. Relative fitness of the *t* haplotype thus combines paternity share with segregation distortion. Transmission of the *t* from +/*t* males was assumed at 0.9 as estimated for this laboratory population elsewhere (LINDHOLM ET AL., 2013).

2.5 DISCUSSION

In controlled sperm competition trials, we confirmed that +/*t* males and the *t* haplotype are highly disadvantaged in the first-to-mate role, which is in strong contrast with the first male precedence when wild type male house mice compete. We expected that this genetically determined sperm precedence inversion would have favoured the evolution of differences in male copulatory or morphological traits that could correspond to differences in the incentive for preventing female remating. However, we did not find differences between male genotypes in male copulatory behaviour, body mass, and reproductive tissues that were consistent with our expectations. In investigating the mechanistic basis of sperm precedence inversion, we found that the *t* haplotype decreased linear sperm velocity and showed signs of premature hypermotility, but had no significant adverse effect on sperm numbers or motility.

2.5.1 The t *haplotype inverses sperm precedence*

Sperm precedence strongly depended on the competing males' genotype at the *t* locus. When wild type brothers competed, we found the first male advantage (model prediction for $P_2 = 0.15$) that is typical for house mice (LEVINE, 1967; FIRMAN & SIMMONS, 2008A). However, when females were mated to two +/*t* brothers, second males obtained the majority of the paternity share (predicted $P_2 = 0.93$). By including detailed observations on the number and timing of ejaculations, we were able to largely rule out the possibility that this sperm precedence reversal was an experimental artefact. In a previous experiment we had shown that when accounting for the number of ejaculations, there was no order effect in sperm competition between +/*t* and +/+ males (CHAPTER 1). Our current experiment confirmed that +/*t* males are drastically disadvantaged when first-to-mate irrespective of the genotype of the second-to-mate male.

Males carrying driving elements are commonly disadvantaged in sperm competition against wild type males (Price & Wedell, 2008; Chapter 1). Price et al. (2008a) showed that *Drosophila pseudoobscura* males carrying a driving X chromosome obtained a very small paternity share when second-to-mate ($P_2 = 0.14$) instead of the typical second male sperm precedence ($P_2 \approx 0.8$; Turner & Anderson, 1984). When first-to-mate, they performed similarly to wild type males ($P_1 = 0.35$; Price et al., 2008a). Intriguingly, sperm precedence in *D. pseudoobscura* changes to extreme first male precedence when sperm are stored for long time periods (Giraldo-Perez et al., 2016). Other species with male drive show variable patterns. Driver males are equally disadvantaged in both mating roles in *Drosophila simulans* (Atlan et al., 2004) and the stalk-eyed fly *Teleopsis whitei* (Wilkinson & Fry, 2001). These examples demonstrate that more species need to be investigated to identify common effects of male drive on sperm precedence.

2.5.2 Consequences for male reproductive tactics?

Over the long evolutionary history of the *t* haplotype (Morita et al., 1992),], the genetically determined difference in defensive sperm competitiveness between +/+ and +/*t* males could have led to genetically determined alternative reproductive tactics. Sperm precedence patterns are predicted to strongly influence the pay-off of mate guarding, with last male precedence generally favouring male mate guarding (Parker, 1974; Alcock, 1994; Alonzo & Warner, 2000; Harts & Kokko, 2013). For example, Sherman (1989) concluded that mate guarding appeared evolutionarily stable in a ground squirrel species with last male precedence, whereas resuming searching for additional females after copulation was inferred as the stable strategy for another ground squirrel species with first male precedence (Sherman, 1989). Given the strong difference between sperm competitiveness in the defensive mating role, the pay-offs of male tactics to reduce or prevent female remating should differ drastically between +/+ and +/*t* males. Thus, +/*t* males should have a strong evolutionary incentive to prevent female remating, because of the large paternity loss. In contrast, +/+ males are strong defensive sperm competitors, and consequently have little to lose after inseminating a previously unmated female. Table 2.3 illustrates how with imperfect prevention of female remating, +/*t* males can only make the best of a bad job. However, when considering fitness at the gene level, partially efficient prevention of female remating could lead to equal fitness between the *t* haplotype and its + counterpart when the transmission advantage balances the paternity loss due to sperm competition. Another potential consequence of the combination of sperm precedence patterns and transmission distortion is that the *t* haplotype might create a higher incentive for males to mate with previously mated females (Table 2.3). However, the pay-offs in this scenario will depend more strongly on the rival male genotype and whether the female will mate with yet another male, and the fitness benefit is limited to *t*-linked genes. Moreover, we have previously reported that copulatory behaviour did not differ between +/*t* and +/+ males when second-to-mate (Chapter 5).

In order to explore the possibility of male alternative reproductive tactics related to the *t* haplotype, we investigated a variety of behavioural and morphological traits within our experimental setting. We hypothesised that prolonged copulation or repeated ejaculation with the same female could serve as a form of mate guarding (Huck & Lisk, 1986; Preston & Stockley, 2006; Raveh et al., 2011), and consequently, that +/*t* males would attempt to prolong copulation or to reduce postejaculatory interval compared to +/+ males. Additionally, +/*t* males' ejaculates may be more competitive closer to the time of ovulation and thus later in oestrus (see below). The difference in sperm competitiveness between +/*t* and +/+ ejaculates could also result in the two

genotypes experiencing different pay-offs from resource allocation towards sperm versus alternative fitness-enhancing features (Engqvist, 2012). Here, we indirectly assessed male investment into scent marking, sperm production and copulatory plug production by measuring the weights of preputial glands, testes and epididymides, and seminal vesicles and coagulating glands. Collectively, we found no evidence for different reproductive tactics in $+/t$ and $+/+$ males. The only trait that showed a significant difference between $+/t$ and $+/+$ males was the weight of seminal vesicles and coagulating glands, but the lower weight in $+/t$ males was opposite to what we had predicted based on the involvement of copulatory plug size in passive mate guarding (Chapter 5). Males are limited in seminal fluids when ejaculating repeatedly (Chapter 4), but whether $+/t$ males become limited more quickly as a function of smaller glands is currently unknown.

Several factors may explain why we did not find any of the hypothesised adaptations to low sperm competitiveness in $+/t$ males. First, our experimental setting may not have reflected a setting in which males exhibit their different tactics. The behavioural traits we measured are likely to be highly phenotypically plastic and males may have behaved simply in accordance with the experimental conditions. For example, preferential allocation to mate acquisition and retention may only be expressed when directly interacting with other males, where $+/t$ males may invest more into suppressing competitors (DeFries & McClearn, 1970). However, previous research in semi-natural settings has produced contrasting results, with $+/t$ males being either more (Lenington et al., 1996) or less (Carroll et al., 2004) socially dominant. With regards to the morphological features measured, differences in resource allocation along trade-offs between pre- and postcopulatory traits may only be discovered when resources are limited (Mehlis et al., 2015). Second, the efficacy of mate guarding is a strong determinant for male tactic pay-offs (Alonzo & Warner, 2000). If females benefit from polyandry, sexual conflict over remating may prevent efficient mate guarding (Stockley, 1997; Alonzo, 2008). The *t* haplotype present in our population is, like many other *t* haplotypes (Klein et al., 1984), associated with embryonic lethal effects, resulting in strong genetic incompatibility between *t* heterozygous mating partners (Lindholm et al., 2013). Polyandry can strongly reduce the cost of this genetic incompatibility (Chapter 1).]. Thus, sexual conflict might limit the possibility for $+/t$ males to prevent female remating. P_2 was slightly higher for $+/t$ than for $+/+$ females, potentially indicating that $+/t$ females discriminate in general against first-to-mate males. However, the biological meaning of this is unclear. Here, both mates had the same genotype at the *t* locus and there was thus no fitness benefit to biasing P_2. Moreover, we found no evidence for cryptic female choice in sperm competition trials involving $+/+$ versus $+/t$ males (Chapter 1). More experiments are needed to elucidate the influence of female choice on the *t* haplotype (Lenington, 1991; Lindholm et al., 2013; Manser et al., 2015). Similarly to conflict between the sexes, constraints arising from male-male competition might affect ejaculation timing. Delaying ejaculation relative to ovulation may be too risky for $+/t$ males under the threat of a take-over by a rival. Male mice respond to the proximity of a rival by premature ejaculation, possibly an adaptation to the risk of take-overs in natural contexts (Preston & Stockley, 2006). We kept all experimental mice in the same room, and thus olfactory and auditory cues may have created a perceived risk of take-over. Third, a model on ejaculate expenditure predicts that the adaptive difference between intrinsically subfertile males and strong sperm competitors not only depends on the difference in sperm competitiveness between the males, but is also sensitive to the frequency of subfertile males and the level of polyandry (Engqvist, 2012). House mice show strong temporal and spatial variation in density (Berry, 1981), with potential consequences for

variation in polyandry levels (Dean et al., 2006) and *t* haplotype frequencies (Ardlie & Silver, 1998; Manser et al., 2011). This means that optimal resource allocation becomes a moving target, and that selection should favour phenotypic plasticity rather than fixed tactics for +/*t* and +/+ males (Cornwallis & Uller, 2010).]. Our study suggests that the pay-off to first-to-mate males does not depend on the frequency of +/*t* males, since P_1 is largely independent of the rival male's genotype. In a natural population, the pay-offs of different tactics will likely depend on a number of additional factors such as the adult sex ratio, female mating rates and male mating capacity, the frequency of males employing a mate guarding tactic, and male control over female remating (Dunham & Rudolf, 2009; Fromhage, 2012). Combining theoretical models with more empirical data from natural populations would be needed to address the evolutionary plausibility of our predictions more quantitatively. Fourth, the investigated traits – copulatory behaviour and resource allocation to reproductive organs – are likely highly polygenic. As such, the *t* haplotype may exert only limited control. As highlighted above, despite the old evolutionary age of the *t* haplotype (Morita et al., 1992), temporal and spatial variation may have prevented the stability in selection required to build-up epistatic interactions between the *t* haplotype and the many non-linked genes underlying these polygenic traits. The genetic architecture underlying the traits under selection may impose strong constraints on the evolution of alternative phenotypes (Brockmann & Taborsky, 2008).

2.5.3 Can sperm characteristics explain sperm precedence?

We investigated sperm features over an extended period of *in vitro* incubation in an attempt to find a proximate explanation for the disadvantage of +/*t* males in defensive sperm competition. Our sperm measurements showed that sperm movement patterns differed significantly between the two genotypes. Sperm from +/*t* males showed lower progressive speed (smaller PC1 values) over the whole incubation period investigated. In contrast, sperm head speed was initially not different between +/*t* and +/+ males, but tended to decrease faster for +/*t* males ($p = 0.096$ for the interaction between *t* haplotype and incubation time). Previous studies have shown a decrease in progressiveness using *t* haplotypes that had been introgressed into laboratory strain backgrounds (reviewed in Olds-Clarke, 1997). Furthermore, the negative effect of the *t* haplotype on average progressiveness without affecting average initial head speed that we found here is in line with several studies that have found premature hypermotility in sperm from +/*t* males measured as *in vitro* movement and ova penetration (Olds-Clarke & Carey, 1978; Olds-Clarke, 1989; Olds-Clarke & Johnson, 1993), *in vivo* sperm movement and transport (Tessler & Olds-Clarke, 1981; Olds-Clarke, 1986), and indirectly from a higher metabolic rate (Ginsberg & Hillman, 1974). Hypermotility is characterised by high curvilinear velocity and low straight-line velocity (Goodson et al., 2011), roughly corresponding to high PC2 values and low PC1 values as found for +/*t* males in the early phases of *in vitro* incubation.

The tendency for +/*t* males to have larger numbers of sperm and higher proportions of motile sperm (Table S5) than +/+ males may be related to our incubation method and the premature hyperactivation exhibited by sperm from +/*t* males. Before incubation, epididymides were cut gently and sperm were required to swim out into the medium within 10 minutes. Our method may thus have selected sperm with more vigorous and non-linear movement. Also, the medium we used represented a benign environment that can sustain high sperm motility over time (Goodson et al., 2012). Natural ejaculates can behave quite differently from epididymal sperm (Li et al., 2015), and *in vivo* conditions impose strong selection on ejaculate quality (Suarez & Pacey, 2006).

In vivo, the premature hypermotility in $+/t$ ejaculates is a likely candidate for the weak sperm competitiveness of $+/t$ males and the sperm precedence inversion found in our experiment. In mammals, activation results in hypermotile sperm, which is crucial for fertilisation and is usually triggered in the oviduct (Suarez, 2008a). In hamsters and lemurs, there is evidence for an optimal insemination timing relative to ovulation (Huck et al., 1989; Eberle et al., 2007). Our data indicate that the optimal timing may additionally depend on the male's ejaculate features. We hypothesise that wild type ejaculate features are co-adapted with insemination timing such as to maximise fertilisation efficacy, and that $+/t$ ejaculates deviate from those features. Prematurely hyperactivated sperm in $+/t$ ejaculates may over time fall below a minimum threshold movement required to penetrate the ova vestments (Suarez, 2008b). This may become most relevant for first-to-mate males when the time window between insemination and fertilisation is substantial. The interval between coitus and ovulation in laboratory mice is estimated at between 2 and 5 h (Snell & Fekete, 1940; Braden, 1957), and females ovulate towards the beginning of the light phase (Braden, 1957). In our experiment, first-to-mate males ejaculated at 7.3 h $\pm$ 1.5 (mean $\pm$ SD) into the 14 h dark phase, followed by second males 2.0 h $\pm$ 0.8 later. If $+/t$ ejaculates are deficient in maintaining their fertilising potential over the time period between ejaculation and release of ova, this could explain why $+/t$ males obtain a particularly low paternity share in a defensive sperm competition role. Future experiments could experimentally manipulate the timing of ejaculation relative to ovulation and the interval between two rivals' ejaculations to confirm or refine this hypothesis.

2.6 Conclusions

Using experimental sperm competition trials, our study confirms previous findings that $+/t$ males are particularly weak sperm competitors when mating in the first-to-mate role typically favoured in mice. The effects of drive elements on sperm precedence patterns and their transmission advantage highlight their potential for influencing male reproductive tactics (Engqvist, 2012). However, our data on copulatory behaviour and reproductive organs did not support alternative reproductive tactics that would have matched our predictions based on sperm precedence patterns. We show that sperm precedence patterns in house mice change from a first male advantage in wild type males to a second male advantage when two t haplotype carrying males compete. Sperm from $+/t$ males show marked differences in their swimming patterns compared to sperm from $+/+$ males, and may fall below a velocity threshold during short-term storage between insemination and fertilisation.

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CHAPTER 3 NO EVIDENCE FOR FEMALE DISCRIMINATION AGAINST MALE HOUSE MICE CARRYING A SELFISH GENETIC ELEMENT

3.1 ABSTRACT

Meiotic drivers distort transmission to the next generation in their favour, with detrimental effects on the fitness of their homologues and the rest of the genome. Male carriers of meiotic drivers commonly inflict costs on their mates through genetic incompatibility, reduced fecundity or biased brood sex ratios. Given these costs, evidence for female discrimination against male carriers is surprisingly rare. One of few examples is the *t* haplotype in house mice, a meiotic driver that shows strong transmission distortion in males and is typically homozygote lethal. As a consequence, mating between two *t* heterozygous (+/*t*) mice leads to high embryo mortality. Previous experiments showing that +/*t* females avoid this incompatibility cost by preferring +/+ versus +/*t* males have inferred preference based on olfactory cues or brief social interactions. Evidence from mating contexts in laboratory settings and semi-natural populations has been inconclusive. Here, we investigated female choice from a large number of no-choice mating trials. We found no evidence for discrimination against +/*t* males based on mating, remating and copulatory behaviour. Further, we found no evidence for avoidance of incompatibility through selective interactions between gametes. The likelihood of mating showed significant effects of female weight and genotype, suggesting that our test paradigm enabled females to exhibit mate choice. We discuss the strengths and limitations of our approach. By explicitly considering selection at both the individual and gene level, we argue why precopulatory female discrimination by +/*t* females may be less evolutionarily stable than discrimination by all females based on postcopulatory mechanisms.

Key words: t haplotype, segregation distortion, indirect benefits, mate choice, female preference, cryptic female choice

3.2 Introduction

Female mate choice for genetic benefits is a much-debated topic in evolutionary biology (Kokko et al., 2006; Kuijper et al., 2012; Hughes, 2015). Several hypotheses regarding the evolution of female preferences for heritable male traits have been formulated, some of which posit that choosy females benefit through producing attractive offspring (Fisherian runaway selection), while others propose that offspring inherit 'good genes' from males that display preferred secondary sexual traits (Andersson & Simmons, 2006). A further potential genetic (indirect) benefit of female preference is producing offspring with compatible alleles, where the genetic quality of the offspring depends on the genetic interactions of the parents' alleles (Kempenaers, 2007; Puurtinen et al., 2009). The different kinds of genetic benefits and direct non-genetic benefits are not mutually exclusive (Kokko et al., 2006), and the distinction between 'good alleles' and 'compatible alleles' (Kempenaers, 2007) might not even be useful, since the frequency of an allele in a population partly determines its additive and non-additive components of genetic variance (Puurtinen et al., 2009). One of the challenges with regards to explaining the evolution of mate choice for genetic benefits is that directional preference should—but empirically does not—lead to the depletion of the genetic variation in the target of the preference (the 'lek paradox'; Kirkpatrick & Ryan, 1991). Several solutions to the lek paradox have been proposed, some of which rely on continuous generation of variation in genetic quality through deleterious mutations (Iwasa et al., 1991). Preference for 'good' genes may thus be seen as discrimination against 'bad' genes (Hughes, 2015), where females avoid mating with males carrying deleterious alleles.

Genomic conflict is a potentially ubiquitous source of variation in genetic quality and compatibility (Burt & Trivers, 2006). Selfish genetic elements undermine otherwise fair inheritance and promote their own success at the cost of the rest of the genome (Burt & Trivers, 2006). Segregation distorters are selfish genetic elements that manipulate meiosis or post-meiotic stages of gamete production, thus exhibiting 'meiotic drive' (Lindholm et al., 2016). Meiotic drive frequently targets male gametogenesis (Taylor & Ingvarsson, 2003), presumably because male gametes are produced in excess and fast cell proliferation in spermatogenesis is under less control than oogenesis (Price & Wedell, 2008). Mating with male carriers of such meiotic drivers can incur a variety of costs to females, either through reduced fertility owing to the elimination of a large proportion of the gametes (Price & Wedell, 2008), to the production of sex-biased broods in the context of sex ratio distorters (Jaenike, 2001), or due to genetic incompatibility between deleterious mutations located on the driver (Zeh & Zeh, 1996). Female preference for males that do not carry meiotic drivers can be expected to evolve in order to avoid these fitness costs (Lande & Wilkinson, 1999; Manser, 2015). There are well-known empirical examples for female discrimination against drive-bearing males (Lenington et al., 1992; Wilkinson et al., 1998), but the evidence available so far indicates that precopulatory female discrimination is not a common strategy for avoiding the costs imposed by selfish genetic elements (Jaenike, 2001; Price & Wedell, 2008; Price et al., 2012; Wedell, 2013). One issue is that any male trait used by females to detect drive males needs to be tightly genetically linked to the drive locus to prevent recombination from breaking up the association between trait and driver (Lande & Wilkinson, 1999; Manser, 2015). In one of the prominent examples of female preference for driver-free males, sex ratio drive in stalk-eyed flies (Wilkinson et al., 1998), male eye span has been identified as the target of female preference (Wilkinson et al., 1998; Cotton et

al., 2014), and is influenced by a locus in the genomic region of the driver where recombination is strongly reduced (Johns et al., 2005). Thus, eye span represents an honest trait that females can use to avoid fertilisation by males with a costly sex ratio distorter. Here, we focus on the second prominent example for precopulatory discrimination against driver males, the *t* haplotype in house mice, where the evidence is less conclusive than in the stalk-eyed flies example.

The *t* haplotype is an autosomal meiotic drive element that shows strong drive in males and normal transmission in females (Ardlie & Silver, 1996; Lindholm et al., 2013). Drive occurs through an elaborate 'poison-antidote' mechanism that impairs the motility of sperm not carrying the *t* haplotype within a +/*t* male's ejaculate and thus gives *t* bearing sperm an advantage in intra-ejaculate sperm competition (reviewed in Herrmann & Bauer, 2012). Several major chromosome inversions provide tight genetic linkage of the *t* haplotype and strongly reduce recombination (Figueroa et al., 1985). Probably as a direct consequence of a build-up of mutations, many *t* haplotypes carry homozygote embryonic lethal alleles (Bennett, 1975). The combination of strong male drive and homozygote embryo lethality makes +/*t* individuals genetically incompatible partners: litter size of +/*t* females mated to +/*t* males is much smaller than in other crosses (Lindholm et al., 2013), giving +/*t* females a strong evolutionary incentive to avoid fertilisation by +/*t* males. Females heterozygous for the *t* haplotype (+/*t* females) have been repeatedly shown to prefer the odour of wild type males (+/+) over +/*t* males (Lenington, 1991), though all studies were performed in a single lab that used wild-derived mice from a mixture of populations, some of which harboured *t* haplotype variants (different *t* haplotypes fall into 16 different complementation groups; Klein et al., 1984). The mechanistic basis for olfactory discrimination has not been identified, although the responsible locus was mapped to the *t* haplotype (Lenington et al., 1988a). The major histocompatibility complex (MHC) was thought to offer a promising candidate for olfactory discrimination because several loci are located on the *t* haplotype (individual *t* haplotypes thus carry unique MHC alleles; Figueroa et al., 1985; Lindholm et al., 2013). However, it was empirically excluded as the target of female discrimination through the use of recombinant females that showed olfactory discrimination despite carrying a *t* haplotype with a wild type MHC haplotype (Lenington et al., 1988a). Thus, it remains unknown what exact signal females use to smell the difference between +/*t* and +/+ males.

Importantly, female preference for wild type males has never been shown in an actual mating context (Lenington, 1991). There is some evidence that female social preference has adaptive functions in house mice (Drickamer et al., 2000; Raveh et al., 2014), but three recent studies showed that the correlation between social preference and paternity share is at best moderate (Thonhauser et al., 2013a; Manser et al., 2015; Zala et al., 2015). Instead, females appear to actively mate with multiple males when given the choice (Rolland et al., 2003; Thonhauser et al., 2013a; Manser et al., 2015; Zala et al., 2015). Multiple mating (polyandry) offers a more parsimonious mechanism than precopulatory mate choice because it does not require the presence of a male phenotype that is tightly linked to the drive locus. Instead, postcopulatory processes such as sperm competition (Parker, 1970) or cryptic female choice (Eberhard, 1996) could simply exploit the fact that male meiotic drive is by default associated with ejaculate features (Haig & Bergstrom, 1995). Strong evidence supports the notion that male meiotic drive reduces the sperm competitiveness of its carriers (Price & Wedell, 2008; Price et al., 2008b; Wedell, 2013; Chapter 1), making polyandry a potentially powerful mechanism to avoid fertilisation by male carriers of drive elements (Haig & Bergstrom, 1995; Zeh & Zeh, 1996).

If +/*t* males are indeed discriminated against by +/*t* females through pre- or postcopulatory processes, fertilisation by a +/*t* male may be costly for +/+ females, too, because of investment

into sons that are unattractive at least to part of the population or disadvantaged in postcopulatory competition. A meta-analysis suggested that benefits through sexy sons are more important for driving female preference than benefits through good genes effects (Prokop et al., 2012). Whenever discrimination by +/*t* females is not fully efficient, +/+ females mating with +/*t* males may also have fewer grandchildren due to genetic incompatibility caused by imprecision of their daughter's mating decision. While both good genes and sexy sons benefits may be important, the fitness benefits for the different female genotypes relative to the costs of pre- and postcopulatory mate choice are currently unknown but are crucial for assessing the net fitness of different behavioural strategies (Manser et al., 2015). Evidence for olfactory preference by +/+ females was found in some (Lenington, 1983; Lenington & Egid, 1985) but not in other studies (Coopersmith & Lenington, 1992; Williams & Lenington, 1993). Experiments involving actual mating contexts in conditions ranging from laboratory settings to natural conditions have found some indications for differences between +/*t* and +/+ females (Carroll et al., 2004; Lindholm et al., 2013; Manser et al., 2015), but may have been subject to biases through prenatal or early postnatal mortality. Moreover, these studies and an earlier one (Levine et al., 1980) showed paternity disadvantages for +/*t* males, but were unable to distinguish between pre- and postcopulatory processes. In natural populations, male dominance adds a further confounding factor that influences both male-male competition and female preference (Coopersmith & Lenington, 1992), and the evidence for an effect of the *t* haplotype on male dominance is mixed (Franks & Lenington, 1986; Lenington et al., 1996; Carroll et al., 2004).

While thus far there is evidence for olfactory discrimination against +/*t* males, it remains unclear how olfactory preference translates into precopulatory mate choice, and whether +/*t* females consistently differ from +/+ females. Here, we test female mate choice with respect to the *t* haplotype in an actual mating context. First, we test for female choice of +/*t* and +/+ males in a no-choice test paradigm where females are presented with only one male at a time, and ask whether female genotype at the *t* locus influences the outcome. We use the occurrence of mating and subtler measures of copulatory behaviour to infer female preferences. In a second stage, we ask whether a female's remating is influenced by the genotype of her first mate. Females may be able to recognise a male's genotype by his ejaculate features (Angelard et al., 2008) and may thus show differential remating behaviour dependent on the genetic quality of their first mate (the 'trade-up' hypothesis; Pitcher et al., 2003). Finally, analysing the distribution of embryo genotypes enables us to address the possibility that compatibility choice occurs between gametes (i.e. that *t*-bearing ova choose wild type sperm).

3.3 Material and Methods

For this study, we investigated previously unreported aspects of three laboratory experiments that all followed a similar mating protocol. The first two experiments involved sperm competition trials to assess the effect of the *t* haplotype (Chapter 1) and of the copulatory plug (Chapter 5) on the outcome of postcopulatory competition between two males. In the third experiment, monogamous matings were conducted to validate copulatory plug size variation (Chapter 5). For this study, we expanded our analyses to address questions related to precopulatory female choice and cryptic female choice.

3.3.1 Experimental animals

We used 259 female (mean age ± SD: 103 ± 28 days) and 162 male (79 ± 27 days) wild house mice *Mus musculus domesticus*. Subjects were sexually mature but initially sexually naïve laboratory-born F1 to F3 descendants from a free-living population in Switzerland (König & Lindholm, 2012), from which we introduce individuals into our breeding colony every generation. Mice were kept under standard laboratory conditions at a temperature of 22–24°C under a 14L:10D light regime. The breeding colony was kept under a normal light cycle (lights on at 05:30 CET), with food (laboratory animal diet for mice and rats, no. 3430, Kliba) and water provided ad libitum. Paper towels and cardboard served as enrichment and nest building material. Experimental subjects were descendants of 62 breeding pairs, of which 31 consisted of at least one individual (typically the male) that had been caught in the free living population from which all breeding individuals descended from (König & Lindholm, 2012). Breeding pairs consisted of monogamous pairs of non-sibling +/+ males and either +/+ or +/*t* females, the latter producing on average 50% +/*t* offspring. At the age of 23–28 days, we weaned offspring and kept them in same sex sibling groups in Makrolon Type III cages (23.5 × 39 × 15 cm). We separated male mice at latest when aggression started between brothers and kept them individually in Makrolon Type II cages (18 × 24 × 14 cm). Mating trials were conducted under a reversed 14L:10D regime (lights on at 17:30 CET) in a room separated from the breeding colony. Animals were moved at least two weeks prior to being used in the experiment. We used +/*t* and +/+ males and females and diagnosed their *t* haplotype status before they entered the experiment. An ear punch tissue sample taken at weaning was used for genotyping and individual identification. We extracted DNA by salt-chloroform extraction (Müllenbach et al., 1989) and diagnosed *t* haplotype status as described below (section 3.3.4). The experimenter was blind with respect to genotype during all procedures, including mating trials, video observations, dissections and genotyping. All procedures received ethics approval by the Veterinary Office Kanton Zurich, Switzerland (licence no. 110/2013) and were conducted in accordance with Swiss law.

3.3.2 Mating trials

The protocol for our mating trials has been described previously (Chapters 1 & 5), and was similar in all three pooled experiments We chose sexually receptive females in pro-oestrus or oestrus based on visual appearance of the vagina and/or on a quick microscopic inspection of vaginal smears that were taken with plastic inoculation loops (modified after Byers et al., 2012). Oestrus stage may affect the likelihood of mating and male copulatory behaviour (Preston & Stockley, 2006) and was thus included our analyses we included a categorical account of oestrus stage ('early', 'medium' or 'late' oestrus; Byers et al., 2012). Males and females were weighed to the nearest 0.1g immediately before the start of the trials, which was 1.8 h ± 0.8 (mean ± SD) after the beginning of the 10 h dark phase of the reversed light cycle (lights off at 7:30 CET). Females were paired with a male in his cage under a red light spot after having removed nesting material to facilitate video observation for the quantification of copulatory behaviour. Females were checked every 1-1.5 hours for the presence of a copulatory plug, indicating ejaculation by the male (McGill, 1962). We released the pair into a handling bin and briefly restrained the female to check her vagina for a plug under dim white light, before reintroducing the pair into the cage. Thus, mice were out of their cage for approximately one minute during a check. For the trials of one of the experiments (N = 45), females were sacrificed after their first mating as part of validation of copulatory plug removal methodology (Chapter 5 For the remaining mated females (N = 170), the plug was then

either removed or left intact (Chapters 1 & 5), after which the female was paired with the second male and checked every 30–60 minutes until either a second copulatory plug was observed or until the beginning of the next dark phase. After the second mating, the plug was again either removed or left intact. Thus, females either had both or neither of their mates' plugs removed. Mated females were kept in isolation with nesting material and ad libitum food and water. Trials in which no plug by the first male was detected were stopped at the end of the dark phase and females were re-tested on a later occasion. Males were sexually rested for a minimum of three days after a trial with mating to allow sperm and seminal fluid replenishment (Chapter 4). Whenever possible, we used full brothers from the same litter (65/70 male pairs) for sperm competition trials to minimise the influence of genetic background and potential maternal effects on mating behaviour and sperm competitiveness.

3.3.3 Copulatory behaviour

Copulatory behaviour in house mice is characterized by initial mounts, a variable number of mounts with intromission (during which the male inserts his penis and performs pelvic thrusts), and ejaculation including the deposition of the copulatory plug (McGill, 1962). One copulatory series includes all mounts and intromissions and ends with ejaculation. Here, we recorded (i) the latency from introduction of the pair into the cage to the first mount (mount latency), (ii) the latency from the first copulatory mount to ejaculation (ejaculation latency), and (iii) the in copula duration at ejaculation as potential indicators of a female's willingness to mate. We also used video recordings to confirm ejaculation by the second male.

3.3.4 Postcopulatory aspects

We sacrificed females 9 days (± 1 day) post coitum using gradual CO2 filling in their home cage and dissected females to retrieve implanted embryos. By doing so we avoided potential biases in the distribution of *t* genotypes due to early post-implantation embryonic mortality associated with the *t* haplotype (*t*/*t* embryos are resorbed in utero; Lindholm et al., 2013; Chapter 1). Embryo viability and paternity results are described elsewhere (Chapters 1 & 5). Here, we further genotyped the Hba-ps4 locus that is located in the genomic region of the *t* haplotype (Schimenti & Hammer, 1990; Lindholm et al., 2013) to obtain data on embryo genotype frequencies (+/+, +/*t* and *t*/*t*) for *t* haplotype drive estimates and questions related to cryptic female choice with respect to gamete genotype.

3.3.5 Statistical analyses

An overview of the sample sizes available for the different analyses is given in Table 3.1. Data will be made available on Dryad upon acceptance of the manuscript.

Using the functions lmer and glmer in lme4 (Bates et al., 2014) in R version 3.1.3 (R Core Team, 2015), we analysed data on mating and remating, copulatory behaviour, and offspring genotypes with generalised linear mixed models (GLMM) and linear mixed models (LMM), depending on the response variable. We compared full models to null models using likelihood ratio tests to test the global null hypothesis that none of the predictors has a significant effect on the response variable, and extracted effect sizes from full models to avoid biasing effect sizes through removal of non-significant terms (Forstmeier & Schielzeth, 2011). Continuous input variables were standardised to a mean of 0 and a standard deviation of 1 to improve interpretability (Schielzeth, 2010). Because many females were re-tested if they did not mate and because all males were used

in multiple trials, we included the identity of the individuals as random effects in all models to account for multiple testing and avoid pseudoreplication. To account for the family structure inherent in our breeding design, we also included female and male parental origin as random effects. We obtained approximate 95% confidence intervals (c.i.) for fixed effects by multiplying Student's t-values for our sample sizes by the standard errors of the predicted values (Crawley, 2007).

Controlling for relatedness – We generally controlled for relatedness between females and males by mating females to two males that were full brothers but not closely related to the female. However, in 5/488 trials females were accidentally paired with a full sibling from a different litter. Moreover, due to our within-population breeding design with a limited number of breeding pairs with overlapping generations, mating trials would by chance be staged between second-degree relatives (such as cousins). To include relatedness in our analyses, we included information from our breeding pedigree, where individuals not sharing any relatives in the two previous generations were assumed to be unrelated. Relatedness estimates thus ranged between zero (no shared grandparents) and 0.5 (full siblings).

Mating trials – First, we analysed mating success (whether or not a plug was detected in a mating trial) with binomial GLMMs. The full model included the following fixed effects: male and female genotype at the *t* locus and their interaction, male and female body weight and their interaction, female age, oestrus stage (categorical variable with three levels), and the pedigree-based relatedness between the two individuals (see above).

Second, we asked whether the genotype of a female's first mate influenced her remating likelihood. We analysed female remating similarly to mating success, here based on video observations. We included the following variables as fixed effects in a binomial GLMM: the genotypes of a female and her first mate as well as their interaction, female and male weight and their interaction, and relatedness between the female and the male. Since in one of the experiments, some of the first males' copulatory plugs had been removed (Chapter 5), we included plug removal as a categorical fixed effect with two levels.

Copulatory behaviour – We analysed three components of copulatory behaviour (mount latency, ejaculation latency and in copula duration at ejaculation) individually using LMMs. Full models contained female and male genotype and their interaction, female and male body weight and their interaction, oestrus stage and relatedness as fixed effects.

Postcopulatory aspects – Paternity outcomes have been published elsewhere and showed no evidence for an influence of female genotype on the sperm competition disadvantage of +/*t* males (Chapter 1). Here, we investigated potential within-ejaculate discrimination at the gamete level, i.e., whether penetration of *t*-bearing ova was non-random with respect to sperm genotype. The proportions of different genotypes of a female's embryos were analysed using binomial GLMMs. In these models, we only included female and male identity as random effects, since family-associated variances showed to be negligible. Significance of genotypic frequency estimates was assessed by comparing approximate 95% confidence intervals to null hypotheses based on previous estimates of transmission in males and females for this population (Lindholm et al., 2013) and on random gamete interactions. Mating order of the sire was included as a covariate to test for a change in the strength of drive with mating order (i.e., timing of ejaculation relative to ovulation) as suggested from work on delayed matings (Braden, 1958) and postpartum oestrus matings (Lenington & Heisler, 1991).

Table 3.1

	+/+ females				+/*t* females				Total
First mate	+/+		+/t		+/+		+/t		
Paired with male	151		145		107		85		488
Mated	71		69		40		35		215
Copulatory behaviour	46		35		24		19		124
Second mate	+/+	+/*t*	+/+	+/*t*	+/+	+/*t*	+/+	+/*t*	
Paired with male	27	30	39	16	14	18	17	9	170
Remated	19	21	29	14	12	12	13	7	127
Sire genotype	+/+		+/*t*		+/+		+/*t*		
Sire mating order	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**	
Embryo genotypes	263	201	87	84	149	95	38	39	956

Overview of sample sizes available for the different analyses (mating, copulatory behaviour, remating and embryo genotypes).

3.4 Results

3.4.1 Mating trials

The three experiments were conducted over the course of almost two years from January 2013 to December 2014, but initial inspection showed that mating success was not significantly different between the three experiments and they were subsequently pooled. In 488 mating trials, 215 females mated as indicated by the deposition of a copulatory plug. Individual females that mated did so after 1.8 ± 1.3 trials (mean ± SD; range 1–8). Females that never mated before the end of the experiments were tested 2.1 ± 1.4 times (range 1–9). In successful trials that led to ejaculation by the male, pairs were separated after 5.7 ± 1.6 h (range 1.5–9.5 h). Pairs that had not mated were separated after 8.5 ± 0.7 h (range 7–11 h).

We analysed mating as a binary outcome in a full model including 389 trials with all information available. Due to our full model approach, trials with missing information regarding any of the predictor variables—most commonly oestrus stage, male body weight and relatedness—had to be excluded. Inspection of the full model showed significant effects of female genotype and female weight (Table 3.2 & Figure 3.1a).). Thus, +/*t* females had a lower likelihood of mating (GLMM: 389 trials, 226 females, 117 males; b [95% c.i.] = 0.55 [1.05, 0.05], z = 2.17, $P = 0.030$), and heavier females were more likely to mate (0.45 [0.19, 0.72], z = 3.36, $P < 0.001$). There was neither a significant main effect of male genotype ($P = 0.550$), nor was the interaction with female genotype significant ($P = 0.645$; Figure 3.1b). These results were robust to a more conservative controlling for multiple testing of individual females, as a model including only each female's first mating trial (GLMM on 207 trials including 104 males) showed very similar results. Thus, the positive effect of female weight on mating likelihood was not driven simply by re-testing females that had not mated at a younger age and had gained weight as time progressed.

Whether or not a mated female remated with her second mate was not significantly affected by any of the variables investigated, including female and male genotype and its interaction. Thus,

the null hypothesis for the full model could not be rejected (GLMM: 145 trials, 84 males; $P = 0.194$; Table 3.3).

3.4.2 Copulatory behaviour

We analysed mount latency, ejaculation latency and in copula duration at ejaculation to look for more cryptic signs of female mate choice. The null hypotheses for the full models on each of the three aspects of copulatory behaviour could not be rejected (LMMs: 108 trials, 61 males; $P = 0.983$, $P = 0.716$ and $P = 0.925$). Thus, copulatory behaviour was not significantly influenced by any of the variables investigated (Figure 3.2 & Table 3.3).

3.4.3 Postcopulatory effects

Figure 3.3 depicts the predicted and empirical genotypic frequencies for the different crosses. Our estimate for male drive from matings between $+/t$ males and $+/+$ females was 0.94 [0.87, 0.97], not significantly different from a previous estimate on this population (0.9; LINDHOLM ET AL., 2013). There was no evidence for an influence of mating order on male drive (GLMM: 171 embryos, 31 females, 32 males; $z = 0.998$, $P = 0.318$), meaning that male drive did not differ between males that were first versus second-to-mate (0.90 versus 0.97). Transmission of the *t* haplotype from $+/t$ females did not deviate from Mendelian segregation (0.53 [0.47, 0.60]). Based on 0.9 drive in males and 0.5 transmission in females, the expected distribution of embryo genotypes from $+/t$ x $+/t$ matings was 0.45 *t/t,* 0.5 $+/t$ and 0.05 $+/+$. Our empirical estimates matched this prediction well: 0.44 *t/t* [0.29, 0.61], 0.56 $+/t$ [0.43, 0.67] and 0 $+/+$. Again, order had no significant effect on this distribution (GLMM: 77 embryos, 15 females, 11 males; $z = 0.202$, $P = 0.840$). Overall, we found no evidence for a reduced transmission of the *t* haplotype in matings between genetically incompatible partners, and thus no influence of female genotype at the *t* locus on drive (cf LINDHOLM ET AL., 2013)

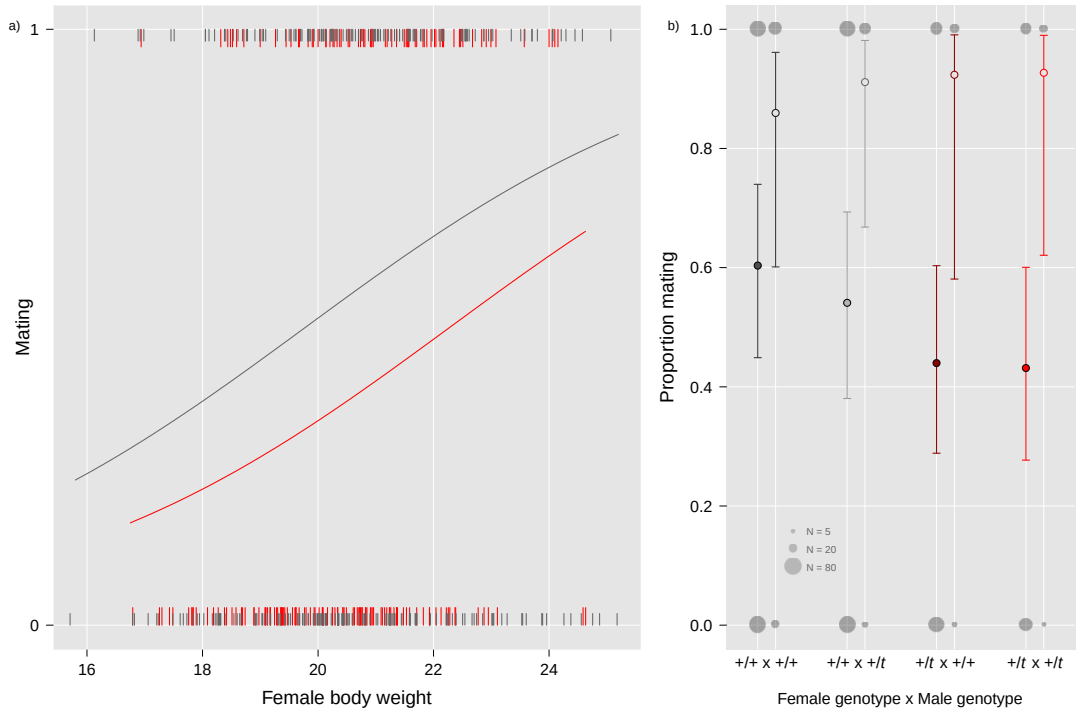

Figure 3.1

a) Mating likelihood as a function of female weight and genotype. Mating likelihood of females increased with their weight and was higher for +/+ than for +/*t* females (see Table 3.2). Ticks correspond to individual mating trials, lines and shaded areas show predictions and approximate 95% CI from a full GLMM. +/*t* females are shown in red, +/+ females in grey.

b) No evidence for discrimination against +/*t* males by +/+ and +/*t* females. Circles and error bars depict mean and approximate 95% CI from full GLMMs on mating likelihood for first matings (solid circles) and rematings (open circles), dependent on female genotype and the genotype of first mates. Raw data are shown as background grey cricles, with surface area proportional to sample size. Neither mating nor remating likelihood was significantly affected by male genotype or its interaction with female genotype (see main text and Tables 3.2 & 3.3).

Table 3.2

Model	Response variable	Random effects	Fixed effects	Mean (SD)	Fixed effect centred/ standardised?	Estimate [approx. 95% CI]	*z* value/ F value	p
GLMM	Mating success	1|Family/Male ID	Intercept (genotypes centred)	–	–	0.02 [-0.47, 0.51]	0.07	0.941
		1|Family/Female ID	Female *t*haplotype	–	y/n	**-0.55 [-1.05,-0.05]**	-2.17	**0.030**
			Male *t*haplotype	–	y/n	-0.15 [-0.63, 0.34]	-0.59	0.558
			Female weight [g]	20.7 (1.7)	y/y	**0.45 [0.19, 0.72]**	3.36	**< 0.001**
			Male weight [g]	25.2 (2.0)	y/y	0.10 [-0.14, 0.35]	0.83	0.404
			Female age [d]	108 (29)	y/y	0.09 [-0.18, 0.36]	0.65	0.515
			Relatedness	0.02 (0.08)	n/n	-0.09 [-3.06, 2.88]	-0.06	0.954
			Early oestrus	–	n/n	-0.31 [-0.88, 0.26]	-1.07	0.285
			Late oestrus	–	n/n	0.03 [-0.57, 0.64]	0.11	0.913
			Female x Male *t*haplotype	–	–	0.22 [-0.72, 1.17]	0.46	0.645
			Female x Male weight	–	–	0.05 [-0.20, 0.30]	0.41	0.683

Model summary from a full model on mating success. The intercept was centred for female and for male genotype by assigning values of -0.5 and +0.5 to +/+ and +/*t* individuals, respectively. Thus, the intercept corresponds to an average between +/+ and +/*t* individuals for unrelated individuals with average body weights, with females of average age at an intermediate oestrus stage. *t haplotype* shows the change for +/*t* relative to +/+ individuals. Centred and standardised fixed effects have a mean of zero and a standard deviation of one (Schielzeth, 2010). Approximate 95% confidence intervals were obtained by multiplying Student's t-values for our sample sizes by standard errors of the predicted values (Crawley, 2007). 95% confidence intervals not overlapping zero and p values < 0.05 are highlighted in bold. GLMM = generalised linear mixed model.

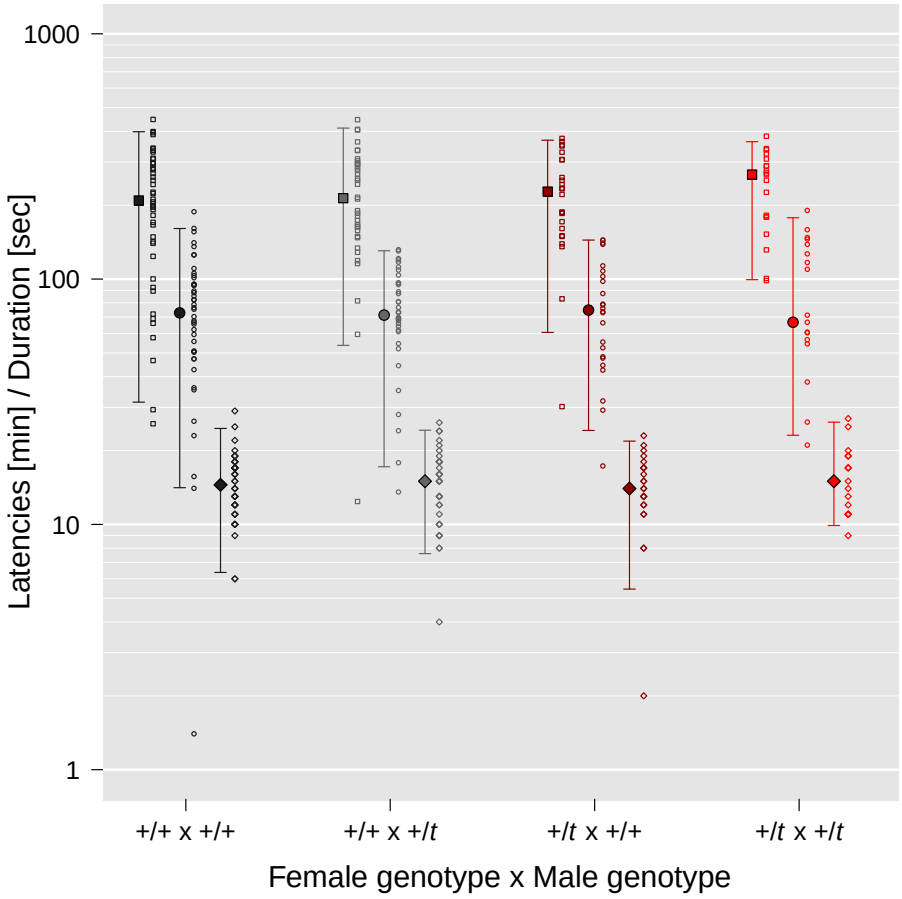

Figure 3.2

Three aspects of copulatory behaviour of first-to-mate males as a function of female and male genotype. Mount latency [minutes; squares], ejaculation latency [minutes; circles] and in copula duration at ejaculation [seconds; diamonds] are shown on a $\log_{10}$-transformed scale for all four possible female x male genotype combinations. Copulatory behaviour was not significantly affected by ay of the variables investigated (Table 3.3). Small symbols represent raw data. Large symbols and error bars show median and 95% quantiles of the raw data. Ejaculation latencies of less than one minute were treated as outliers and thus excluded.

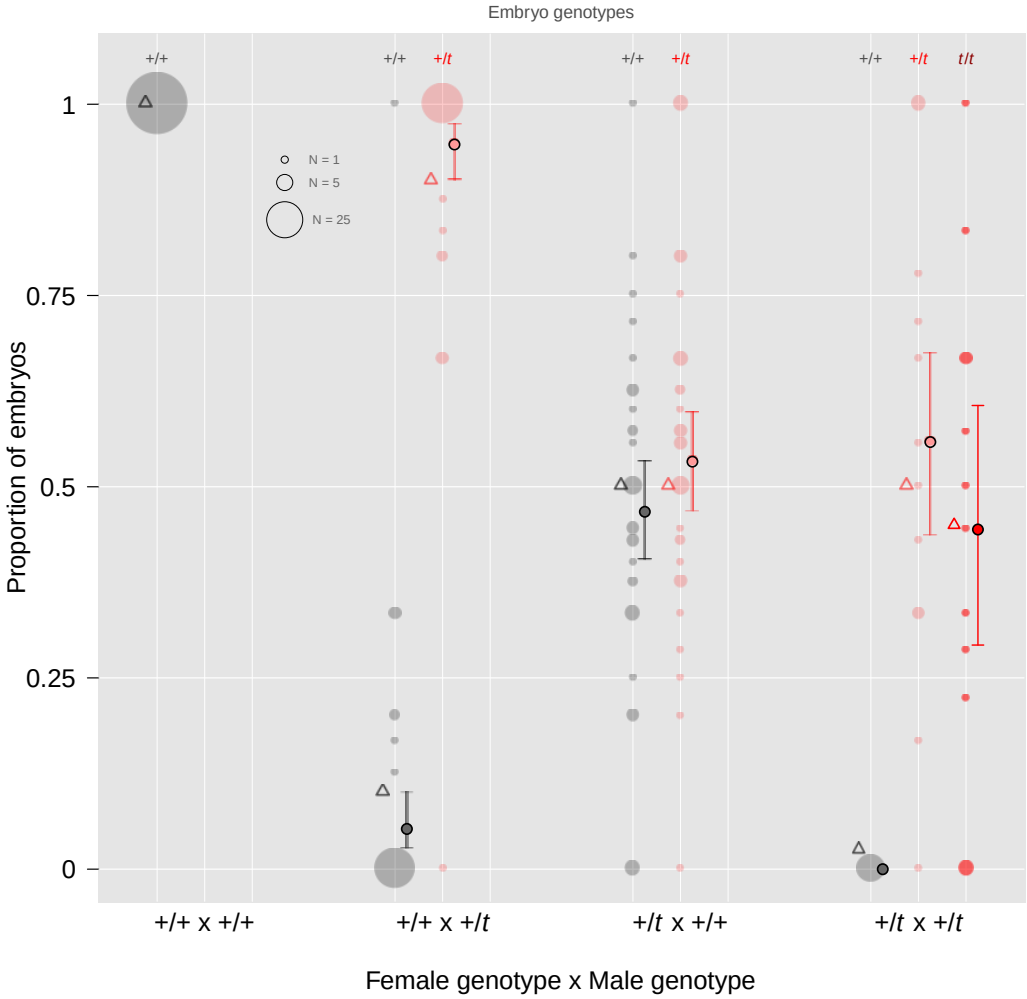

Figure 3.3

Distribution of embryo genotypes and estimates of male and female *t* transmission in for different parental genotype combinations. Circles and error bars show predicted mean and approximate 95% CI for embryo genotype frequencies from GLMMs. The parental genotypes are indicated on the X-axis, embryo genotypes are indicated by colours, and by symbols at the top of the figure. Triangles show the predicted embryo genotype frequencies based on transmission of the *t* from +/*t* males to 0.9 of their offspring and Mendelian segregation in females as estimated for this laboratory population elsewhere (LINDHOLM ET AL., 2013). There was no evidence for drive reduction or for non-random fusion of sperm and ova in crossings of incompatible genotypes (see main text).

Table 3.3

			Fixed effects		LRT		
Model	**Response variable**	**Random effects**	**Full model**	**Null model**	**χ^2**	**df**	***p***
GLMM	Remating	1|Family/Male ID 1| Female family	Intercept Female *t*haplotype Male *t*haplotype Female weight [g] Male weight [g] Relatedness Plug removal Female x Male *t*haplotype Female x Male weight	Intercept	11.85	9	0.222
LMM	Mount latency	1|Family/Male ID 1|Female family	Intercept (genotypes centred) Female *t*haplotype Male *t*haplotype	Intercept	2.42	9	0.983
LMM	Sqrt(Ejaculation latency)	1|Family/Male ID 1|Female family	Female weight [g] Male weight [g] Relatedness	Intercept	6.24	9	0.716
LMM	In copula at ejaculation	1|Family/Male ID 1|Female family	Early oestrus Late oestrus Female x Male *t*haplotype Female x Male weight	Intercept	3.78	9	0.925

Model summaries on full model tests for remating and copulatory behaviour. Fixed effects were centred and standardised as indicated in Table 3.2 and were the same for all three models of copulatory behaviour. Shown are the results from likelihood ratio tests (LRTs) on the full versus the null model (including only the intercept and random effects). GLMM = generalised linear mixed model, LMM = linear mixed model.

3.5 Discussion

In a large number of controlled mating trials, we found no evidence for female discrimination against male carriers of the *t* haplotype. This was true for precopulatory mate choice, copulatory behaviour and remating. Moreover, we found no evidence for cryptic reduction of drive based on genotypes of embryos retrieved during gestation. These results highlight that precopulatory discrimination against *t* haplotype bearing males may not be a common female strategy to avoid fitness costs associated with this meiotic driver. Female multiple mating offers a more parsimonious and potentially more powerful mechanism.

3.5.1 Precopulatory female preference?

We found no evidence for precopulatory discrimination against +/*t* males, neither by genetically incompatible *+/t* females nor by +/+ females. Our findings contrast with previous studies that have reported consistent preferences by +/*t* females for the airborne scent of +/+ males over that of +/*t* males (Lenington, 1991). Although urine from +/*t* males has been suggested to differ in

volatile chemical profile from wild type males (Jemiolo et al., 1991), female house mice appear to require information from non-volatile of urine to develop preferences for individual males (Ramm et al., 2008; Roberts et al., 2010). Furthermore, some recent studies have suggested that the correlation between social preference and sexual preference (as measured by parentage of offspring) may be weak in wild-derived house mice (Thonhauser et al., 2013a; Manser et al., 2015; Zala et al., 2015). Arena settings have also been used to investigate discrimination against +/*t* males, with mixed results (Lenington, 1983; Franks & Lenington, 1986). The only study so far that allowed females to choose between +/*t* and +/+ males while preventing male-male interactions found no support for precopulatory choice (Manser et al., 2015). The paternity disadvantage of +/*t* males was consistent with purely postcopulatory processes, but the experimental design did not allow for a conclusive distinction between pre- and postcopulatory mechanisms and did not control for female oestrus cycle (Manser et al., 2015). The findings reported here are consistent with females using a strategy that relies on the strong sperm competition disadvantage to +/*t* males (Chapter 1).

Arguably, our assessment of female preference suffers from some limitations that merit discussion. We tested female choice in a laboratory setting, where choice was the outcome of a mating trial that was subject to interactions between female preference and environmental and male effects (Wagner, 1998). Being introduced into and confined in a male's cage, females might have had little chance to resist male coercion and exhibit choice according to their preferences. If male physical coercion influences mating, one might expect a significant positive effect of male weight, either as a main effect or in the interaction with female weight. However, we did not find any influence of male weight on mating outcome (see Table 3.2). Moreover, the significant positive effect of female weight on mating was opposite to that predicted if light females were less able to resist male coercion (Figure 3.1a). We can only speculate on why heavier females were more likely to mate. First, heavier females may have a better ability to carry a pregnancy to full term. Second, females were kept in small same-sex groups where competition between females might have led to dominance interactions and reproductive suppression of subordinate females by heavier dominant females (Stockley et al., 2013). Third, if female fecundity increases with female body weight (Singleton et al., 2001), increased mating by heavier females may have been a product of male choice for heavier females (Dewsbury, 1982). We also found a significant difference in mating likelihood between +/+ and +/*t* females, which may have been caused by male choice for +/+ over +/*t* females, or by a more reactive personality in +/*t* females (activity and exploration: Auclair et al., 2013; trappability: Lenington & Franks, 1985; Drickamer & Lenington, 1995; social dominance and pregnancy likelihood: Franks & Lenington, 1986). Additionally, our observations of copulatory behaviour did not reveal evidence for more subtle expression of female preference, since more resistance against +/*t* males should have increased mount latency, ejaculation latency and/or decreased *in copula* duration at ejaculation. Although all females were presumably in oestrus, the incidence of mating was moderate, but comparable to a recent study that used females from a laboratory strain that is likely to have experienced positive selection on female mating propensity (Ramm & Stockley, 2014). Trials in which mating did not occur could either indicate female and/or male mate choice, or inaccuracy in oestrus detection. Here, in the majority (82%) of the mating trials we detected oestrus using vaginal smears, a method that is well-established for house mice (Byers et al., 2012), making it unlikely that oestrus detection was wrong in more than half of the mating trials and that there would have been an oestrus detection bias towards heavier females and +/+ females. Collectively, our findings suggest that females actively chose to mate rather than simply being forcefully mated, but did not discriminate against +/*t* males.

Sequential stimulus presentation in no-choice test paradigms has been proposed as a more powerful test of female preference than simultaneous stimulus presentation (Wagner, 1998), and latency to copulation has been shown to be a reliable predictor of male mating success in field crickets (Shackleton et al., 2005). Studies in invertebrates and vertebrates (e.g. Maclaren & Rowland, 2006; Rutstein et al., 2007) have established that no-choice tests enable females to exhibit mate preference, but have also highlighted that results and effect sizes can depend on the test paradigm used (for a meta-analysis see Dougherty & Shuker, 2015). Our no-choice test paradigm offered the advantage of removing male-male competition, and the use of full brothers in the vast majority of trials ensured that +/*t* males did not systematically differ from +/+ males in genetic background. However, our mating design did not allow females to simultaneously compare males. Experiments with female brown lemmings *Lemmus trimucronatus* provided some evidence for female discrimination between dominant and defeated males in a no-choice setting, as did a simultaneous choice setting (Huck & Banks, 1982). In house mice, no-choice tests have demonstrated cryptic *male* choice regarding mating likelihood (Ramm & Stockley, 2014), copulatory behaviour (Preston & Stockley, 2006) and ejaculate allocation (Ramm & Stockley, 2007). In the only study to date that directly compared preferences of *female* house mice between simultaneous stimulus presentation and no-choice trials, the authors found that females discriminated against hetero-subspecific males only when allowed to compare males directly, and appeared to mate indiscriminately in no-choice trials (Zinck & Lima, 2013). However, this negative result from no-choice trials was based on a total of twelve trials, of which only four resulted in ejaculation. No-choice tests are associated with smaller effect sizes than simultaneous choice tests (Dougherty & Shuker, 2015), thus Zinck and Lima's (2013) study may have lacked the statistical power to detect more subtle discrimination during no-choice trials. Our large sample size makes it unlikely that our negative result is due to a lack of statistical power. Nevertheless, we cannot rule out that preference in female house is relative and may only be exhibited when more than one potential mate is available.

3.5.2 Is discrimination by +/t females plausible?

Expecting female discrimination against genetically incompatible males in the context of the *t* haplotype is intuitively appealing: genetic incompatibility has strong immediate fitness consequences, and the restriction of compatibility effects to few loci should facilitate the evolution of compatibility mate choice (Puurtinen et al., 2009). Disassortative mating should lead to negative linkage disequilibrium between the preference locus and the drive locus if there is no physical linkage of the preference locus to the *t* haplotype (Manser, 2015). However, the strong linkage between the male signal and the drive locus that is required for stability of female preference (Lande & Wilkinson, 1999; Manser, 2015) is facilitated by major chromosomal inversions that encompass many potential candidate loci (e.g. MHC loci; Lindholm et al., 2013; but see Lenington et al., 1992). On the other hand, there are also good reasons to expect that *t*-specific female preference is not evolutionarily stable. First, the importance of MHC for mate choice in mice remains controversial (Roberts & Gosling, 2003; Sherborne et al., 2007), and may be overridden by the influence of major urinary proteins (MUPs) that are not linked to the *t* haplotype (Krauter et al., 1982). Although choosing males with MHC alleles different from self could lead to discrimination of +/*t* males by +/*t* females, it might also result in potentially maladaptive preference for +/*t* males by +/+ females because they could on average share fewer alleles than with +/+ males. Second, discrimination against the *t* haplotype that is controlled by a locus located on the *t* haplotype may

not be expected to evolve or remain evolutionarily stable. Suppression of selfish genetic elements to resolve genomic conflict is expected to evolve in unlinked genomic regions (Burt & Trivers, 2006). In turn, selection acting on the driving element will favour escaping suppression. Thus, selection will favour driving elements that evade detection by females (Price et al., 2012). Even in females, the situation may not be as clear as stated previously. Lenington and colleagues stated that *"[…] given the deleterious effects of t haplotypes when homozygous, it is possible that more copies of t chromosomes will be transmitted to the next generation if +/t females avoid mating with +/t males"* (Lenington et al., 1988a). We argue that, from the view of the *t* haplotype, even selection acting on the *t* haplotype in females will not favour discrimination against a copy of the same *t* haplotype unless the probability of $+/t$ males inseminating +/+ females was reduced by mating with $+/t$ females, e.g. in a strictly monogamous population. In individual litters, the absolute copy number of lethal *t* haplotypes that are transmitted to the next generation is not decreased when females accept incompatible mates. The offspring carrying *t* haplotype may even benefit from homozygote lethality, if reduced sibling competition increases their individual fitness (Charlesworth, 1994).

While we cannot rule out that avoidance of $+/t$ males can arise on the *t* haplotype (possibly as a by-product of pre-existing female preference loci being located in the genomic region of the *t* haplotype), such a preference is unlikely to be evolutionarily stable.

3.5.3 Alternative ways to avoid meiotic drivers

If precopulatory discrimination against male carriers of selfish genetic elements is indeed rare (Price & Wedell, 2008), how else might females avoid the associated fitness costs? Postcopulatory female choice offers a possibility to select directly on the haploid genotype at the gamete level (Birkhead & Pizzari, 2002). Importantly, unlike other phenotypic correlates of drive that may not reliably indicate the presence of a driver, changes in ejaculate features are a direct and inevitable consequence of drive in males (Haig & Bergstrom, 1995). X-linked sex ratio distortion reduces ejaculate size by killing virtually all of the Y-bearing sperm, offering a plausible mechanism for how females may detect driver males after insemination. Indeed, *Drosophila simulans* females use fewer of their stored sperm for fertilisation and remate more quickly after mating with males carrying a sex ratio distorter than after mating with wild type males (Angelard et al., 2008). The *t* haplotype does not affect ejaculate size but instead more subtly influences sperm motility features (reviewed in Olds-Clarke, 1997), possibly making it more difficult for females to detect $+/t$ males. Here, we found no evidence that remating was affected by a female's first mate, either because females are unable to detect the *t* genotype, or because polyandry is a successful female strategy for avoiding fertilisation by $+/t$ males that is employed equally by $+/t$ and +/+ females (Chapter 1; see below). Nevertheless, there is some evidence from experimentally delayed matings (Braden, 1958) and a comparison between matings during naturally cycling oestrus versus postpartum oestrus (Lenington & Heisler, 1991), indicating that the timing of mating can affect drive, although this tends not to be the case for *t* haplotypes with strong male drive (Yanagisawa et al., 1961). Two previous studies have investigated the distribution of offspring genotypes in crosses between two $+/t$ individuals and have found evidence for selective penetration that resulted in a reduction of drive (Bateman, 1960; Lindholm et al., 2013). Here, we genotyped embryos that we retrieved at an early stage of gestation, thus including *t/t* embryos before resorption. Although we did not directly control the timing of mating and we did not know the timing of ovulation, first-to-mate males on average inseminated females earlier relative to ovulation than second-to-mate males. Our finding that drive was not affected by mating order is

in line with previous work that found no effect of insemination relative to the timing of ovulation for *t* haplotypes with strong male drive (YANAGISAWA ET AL., 1961). Further, we found no evidence for discrimination against *t*-bearing sperm by *t*-bearing ova, as the genotype distribution in embryos from +/*t* females that were sired by +/*t* males matched the expected distribution based on strong male drive and Mendelian inheritance in females. These effects suggest that if females *do* exhibit active postcopulatory discrimination against +/*t* males or against *t*-bearing sperm, +/+ and +/*t* females do so to the same extent (CHAPTER 1; but see LINDHOLM ET AL., 2013). Here, our rather small sample size for fertilisation of +/*t* females' ova by +/*t* males prevents us from drawing firm conclusions. The small sample size was mainly caused by the +/*t* males' strong disadvantage in sperm competition against +/+ males (CHAPTER 1).

Because of the negative effects of male meiotic drive on male fertility and sperm competitiveness (PRICE & WEDELL, 2008; PRICE ET AL., 2008A; CHAPTER 1), inciting sperm competition by mating with multiple males (PARKER, 1970) may offer a simple general mechanism for protection from the harmful effects of drive in males (PRICE ET AL., 2008B; MANSER ET AL., 2011; WEDELL, 2013; HOLMAN ET AL., 2015). Available evidence shows that female house mice are actively polyandrous (ROLLAND ET AL., 2003; THONHAUSER ET AL., 2013A; MANSER ET AL., 2015) and that multiple mating is considerable in wild populations (DEAN ET AL., 2006). Males carrying the *t* haplotype are strongly disadvantaged in sperm competition (CHAPTER 1), particularly when first-to-mate (CHAPTER 2), suggesting that polyandry is only ineffective when all of a female's mates are *t* heterozygous. Kempenaers (2007) suggested three questions to address when investigating mate choice for good versus compatible genes. The questions focus on a) whether the optimal mate is different for individual females, b) whether there is evidence that females chose accordingly, and c) the mechanistic basis for the choice. In the context of the *t* haplotype, while b) and c) have received some empirical support, we argue that a) has been somewhat neglected. When considering long-term fitness consequences, fertilisation by +/*t* males appears costly to both +/*t* and +/+ females. Polyandry provides a very effective possibility for avoiding fertilisation by costly +/*t* males, both for +/+ and +/*t* females, although the costs of polyandry (e.g., enhanced predation risk, sexually transmitted pathogens; JENNIONS & PETRIE, 2000) will influence the net fitness of this strategy. Importantly, polyandry offers a parsimonious explanation for a mechanism of discrimination that is inherently linked to the locus that inflicts the costs. More research in wild populations is needed to assess the importance of pre- and postcopulatory sexual selection on ecological dynamics of meiotic drive (LINDHOLM ET AL., 2016).

3.6 Acknowledgements

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CHAPTER 4 FUNCTION OF COPULATORY PLUGS IN HOUSE MICE: MATING BEHAVIOR AND PATERNITY OUTCOMES OF RIVAL MALES

4.1 ABSTRACT

Polyandry is widespread across animal taxa and subjects males to intense postcopulatory sexual selection, which favours adaptations that enhance a male's paternity success, either by decreasing the risk of sperm competition and/or by increasing the competitiveness of the ejaculate. Copulatory plugs deposited by males are thought to have evolved in the context of sperm competition. However, experimental studies that assess the function of copulatory plugs remain scarce. Moreover, most studies have used unnatural manipulations, such as ablating plug-producing male glands or interrupting copulations. Here, we investigated whether repeated ejaculation affects plug size in a mammalian model species, the house mouse. When males experience short periods of sexual rest we found that plug size decreased over repeated ejaculations so that time since last ejaculation can be applied as an approximation for plug size. We induced natural variation in plug size arising from variation in male sexual restedness and investigated the behaviour and paternity success of rival males. Male behaviour in the offensive mating role (second) was influenced, albeit not significantly, by the sexual restedness of the first male to mate, and therefore the size of his plug. However, second males sired a significantly greater proportion of embryos when competing against a male that had recently mated compared with a male that had not. This supports a potential role of the plug in promoting a male's competitive fertilization success when remating occurs, which could be mediated both by delaying female remating and by ensuring efficient sperm transport through the female reproductive tract.

Key words: polyandry, sperm competition, copulatory behaviour, sperm depletion, Mus musculus domesticus

4.2 Introduction

When females mate with multiple males during a single reproductive cycle, sperm will often be forced to compete for fertilization (Parker, 1970). Sperm competition is recognized as a strong evolutionary force that selects for males to maximize their reproductive success through increased production of higher-quality sperm (Simmons, 2001). Moreover, postcopulatory competition favours behavioural adaptations that optimize ejaculate allocation among available females (reviewed by Wedell et al., 2002) or that decrease the risk of sperm competition, through the manipulation of female mating behaviour (Gillott, 2003) or mate guarding (Parker, 1970). Copulatory plugs have evolved independently in many different animal taxa, including insects (Matsumoto & Suzuki, 1992), spiders (Masumoto, 1993), reptiles (Devine, 1975) and mammals (Hartung & Dewsbury, 1978; Dixson, 1998), and are thought to obstruct rival males and prevent or delay subsequent inseminations (Parker, 1970).

Empirical support for a role of postcopulatory competition in favouring the evolution of copulatory plugs has come from studies adopting a variety of methodologies and performed on a broad range of taxa (insects: e.g., Orr and Rutowski 1991; Polak et al. 2001; arachnids: e.g., Masumoto 1993; Kunz et al. 2014; snakes: Shine, Olsson, and Mason 2000; rodents: Martan and Shepherd 1976). For example, indirect support comes from comparative studies that have found that plug size correlates negatively with female mating frequency among butterflies (Simmons, 2001) and that relative seminal vesicle size (the accessory glands that produce the proteins that coagulate to form the plug) varies with mating system among primates (Dixson, 1998). Further support comes from studies that show associations between the rates of evolution of coagulating semen components and both relative testis size among rodents (Ramm et al., 2009) and mating system among primates (Dorus et al., 2004). In contrast, several within species studies suggest that the presence of the copulatory plug does not affect female remating behaviour or the outcome of sperm competition (nematodes: Timmermeyer et al. 2010; lizards: Moreira and Birkhead 2003; Moreira et al. 2007; snakes: Friesen et al. 2014; deer mice: Dewsbury 1988a). However, such findings need not counter the hypothesis that copulatory plugs have evolved in response to selection via sperm competition. Given the many potential benefits of polyandry (Jennions & Petrie, 2000), females are expected to counteract male attempts to prevent remating (Stockley, 1997), generating sexual conflict over plug efficacy. Moreover, we should also expect to see complex coevolutionary dynamics between male defensive and offensive adaptations for plugging and plug displacement, respectively (Fromhage, 2012). Intrasexual and intersexual conflicts are expected to generate considerable variation in plug efficacy across taxa at any point in time.

When considering rodent species, previous researchers have concluded that the mating plug is most likely an adaptation arising from postcopulatory competition (reviewed in Voss 1979). It was noted that 1) many rodent species do not form strong pair bonds and females mate polyandrously (Voss, 1979), 2) copulatory plugs are formed exclusively by males, suggesting a potential conflict of interest between the sexes (Koprowski, 1992), 3) rodent plugs are usually very hard, tightly adhering to the vaginal epithelium and thus difficult to remove (Voss, 1979), and 4) plug tenure in the female reproductive tract typically exceeds the time span over which the ova can be fertilized (Voss, 1979). Indirect support for a function of the copulatory plug in rodent sperm

competition comes from a phylogenetically controlled comparative study, which showed that the relative size of seminal vesicles covaries positively with testis size relative to body weight, a widely utilized proxy for the level of sperm competition (Ramm et al. 2005). Within species, studies offer contrasting findings. While in the guinea pig *Cavia porcellus,* the copulatory plug was found to be 100% effective at preventing subsequent mates from siring offspring (Martan and Shepherd 1976), experimental plug removal did not affect paternity share in the deer mouse *Peromyscus maniculatus* (Dewsbury, 1988a).

The ejaculate represents a substantial reproductive investment by males (Dewsbury, 1982) and males can become sperm limited when matings occur frequently or in quick succession (Wedell et al., 2002). However, although sperm depletion over consecutive ejaculations has been investigated in a number of rodents (Huber et al., 1980; Dewsbury & Sawrey, 1984; Austin & Dewsbury, 1986; Pierce et al., 1990), reduction in plug-producing ability has not been widely studied. Many male rodents produce large copulatory plugs that occupy the entire vaginal lumen and thus likely represent a costly investment (Baumgardner et al., 1982). In laboratory rats (*Rattus novegicus*), the size of the copulatory plug decreases across the first 3 ejaculations, despite the fact that sperm numbers remain consistently high (Austin and Dewsbury 1986; but see Tlachi-López et al. 2012 for an opposite effect at the 8th ejaculation). A reduction in plug size across successive matings highlights the potential for the effectiveness of the copulatory plug in preventing subsequent inseminations to vary, dependent on male mating status.

Male house mice produce large copulatory plugs from coagulating proteins that are secreted from both the seminal vesicles and the coagulating glands (Gotterer et al., 1955; Rugh, 1968). Early studies in mice concluded that plug formation was neither necessary nor by itself sufficient for pregnancy (McGill et al., 1968; McGill, 1970), but that stimulation by the male's ejaculatory reflex, prolonged by the copulatory plug, increases the likelihood of pregnancy (McGill & Coughlin, 1970; Leckie et al., 1973). Pang et al. (1979) suggested that the contents of the seminal vesicles and the associated volume of the ejaculate, rather than the plug per se, were crucial to ensure normal fertility. Unfortunately, however, many of the early studies used males whose accessory glands had been removed, making it impossible to rule out pleiotropic effects associated with surgical gland removal. More recently, Dean (2013) demonstrated that females mated to males with a knockout of the transglutaminase IV gene, and hence unable to form a copulatory plug, showed a dramatic reduction in uterine sperm numbers and pregnancy rates. This could be indicative of potential sperm reflux immediately after ejaculation and possibly of reduced vaginal stimulation (Dean, 2013). These results suggest that the copulatory plug is necessary to ensure fertility in mice even in the absence of postcopulatory competition. Nevertheless, depositing a small plug might be sufficient to ensure pregnancy. The benefits of producing a large plug are not well understood and might only be revealed when selective forces arising from competition between males are considered. Multiply sired mouse litters have been documented in nature (Dean et al., 2006; Firman & Simmons, 2008b; Lindholm et al., 2013; Thonhauser et al., 2014) and from sperm competition trials performed in the laboratory (Firman & Simmons, 2008a; Thonhauser et al., 2013b; Manser et al., 2015; Chapter 1). These studies suggest either that plugs are not always deposited or that plugs are ineffective as a chastity enforcement mechanism. Nevertheless, the copulatory plug could benefit its producer if it affected a subsequent competitors' copulatory behaviour in such a way as to delay ejaculation and ensure their rival's sperm reach the fertilization site at a sub-optimal time (Parker, 1970; Ramm et al., 2005). Hence, males that ejaculate at the optimal timing while delaying their competitor's ejaculation via a copulatory plug could benefit from an increased paternity share

(e.g., Coria-Avila et al. 2004; but see Klemme and Firman 2013 for a contradicting finding in house mice). Notably, in house mice, the first male to mate sires the majority of offspring, even when the copulatory plug is experimentally removed (Levine, 1967; Firman & Simmons, 2008a), most likely because males mating in this position ejaculate closest to the time that the ova are released (Gomendio et al., 1998).

Here, we used an experimental approach to assess the role of the copulatory plug in sperm competition in house mice. We used controlled experimental matings to investigate variation in copulatory plug size across repeated ejaculations, and its influence on both the mating behaviour of rival males and the outcome of sperm competition. By doing so, we assessed multiple mechanisms by which the copulatory plug could affect male fitness, from preventing sperm competition altogether, to altering rival male mating behaviour and paternity share.

4.3 Material and Methods

4.3.1 Source populations and experimental animals

Male ($n = 77$) and female ($n = 88$) lab-born house mice (*Mus musculus domesticus*) were fourth- to fifth-generation outbred descendants of wild mice caught on 3 islands located off the coast of Western Australia (Boullanger Island, Whitlock Island and Rat Island; see Firman and Simmons 2008a for details). These populations had previously been shown to differ in levels of multiple paternity (between 17% and 71% of litters) that were correlated with relative testes sizes (Firman & Simmons, 2008b). The mice were kept in standard mouse boxes (groups: 25×40×12 cm; individuals: 16×33×12 cm) on a reversed light-dark cycle (14L:10D) with a temperature of 24 °C and food (Rat and Mouse Pellets, Specialty Feeds) and water provided ad libitum. For all three populations, breeding pairs were housed together until the female was visibly pregnant. Before parturition, mice were separated and housed individually. At three weeks of age, litters were weaned and kept in sibling groups (females) or individually (males). For the first experiment, we used sexually experienced mice between 12 and 14 weeks of age (mean body weight ± standard error [SE] males: 21.0g ± 0.5, females: 19.1g ± 0.4). For the second experiment, we used the offspring of the mice from the first experiment when they were 7–12 weeks old (mean body weight ± SE males: 17.0g ± 0.2, females: 14.3g ± 0.3). Females were all virgins, and males were sexually naive at the start of the experiment.

4.3.2 Plug size over consecutive ejaculations

In the first experiment, we investigated whether the copulatory plug decreased in size across successive ejaculations. We chose pro-oestrus and oestrus females based on the appearance of their vagina (Byers et al., 2012) and placed them in a male's cage. Depending on our appreciation of the stage of oestrus, females were then checked for a copulatory plug approximately every 2 h. Copulatory plugs were removed using a blunt probe (Firman & Simmons 2008b) and weighed to the nearest 0.1mg. A second receptive female was given to the male and again checked every 2 h for a copulatory plug. On detection, these plugs were again removed and weighed. If no second ejaculation was achieved within 3 days, the pair was separated and the male rested for at least 7 days before starting new mating trials with different females. We obtained the weights of first and second plugs for 27 of the 30 males that were included in our paired design.

4.3.3 Effect of plug size on copulatory behaviour and paternity outcome

In the second experiment, we assessed whether sexual restedness influenced rival copulatory behaviour and paternity share (P_2; Figure 4.1). In each trial, a first sexually naive male ($n = 27$) was allocated a sexually receptive female (based on vaginal appearance; BYERS ET AL. 2012) who was checked every 2 h for the presence of a copulatory plug. After ejaculation, the copulatory plug was left intact, and female A was paired with a second male A. The first male, now sexually unrested, was allocated a different female B that was again checked every 2 h for the presence of a plug. Pairs that had not mated were separated at the end of the light cycle and were re-paired at the beginning of the next light cycle. On detection of a copulatory plug produced by the first male, female B was paired with second male B. Thus, we used time between ejaculation with female A and female B as a measure of a first male's sexual restedness. It is important to note that when males are sexually rested for a short period of time, they may become depleted with respect to both sperm and copulatory plug material. To investigate potential mechanical effects of the plug on female remating, we recorded and assessed the mating behaviour of the second males to mate (see below). However, paternity success is likely to be a function of the relative number of sperm in the female reproductive tract (GOMENDIO ET AL., 1998), and thus may be influenced by both sperm and copulatory plug depletion.

Matings performed by the second males were observed remotely via filming with a video camera (Sony DCR-SR40) to obtain behavioural data and to ensure that the males had ejaculated (i.e., ejaculation by a second-male-to-mate cannot be confirmed by the presence/absence of a copulatory plug as the first male's plug is already present). To facilitate remote observation, we transferred second males and soiled bedding from their own cage into transparent boxes (11×18×12 cm) immediately before the beginning of the mating trial. Overall, 52 females mated with a first male and were subsequently paired with a second male. After successful mating trials, females were housed individually and provided with nesting material. Females were euthanized by intraperitoneal injection of Euthal 12–14 days *post coitum*, and embryos were resected and stored in 100% ethanol.

4.3.4 Copulatory behaviour

Copulatory behaviour of male mice is characterized by initial mounts, a variable number of mounts with intromission (during which the male inserts his penis and performs pelvic thrusts), and ejaculation including the deposition of the copulatory plug (MCGILL, 1962). Ejaculation is characterized by an increase in thrust frequency, a final 'shudder' and a phase of immobility, during which the pair often tip over onto their sides (MCGILL, 1962). One copulatory series includes all mounts and intromissions, and ends with an ejaculation. The copulatory behaviour of second-to-mate males was scored from the video recordings. We collected detailed behavioural data from the first copulatory series of second males on 1) the latency from introduction of the female until the first mount, 2) the latency (from first mount) to the first intromission, 3) the number of copulatory bouts (mounts and intromissions) until ejaculation, 4) the latency to ejaculation (from the first mount), and 5) the duration of genital contact during ejaculation. Because males sometimes perform 2 full copulatory series with the same female (ESTEP ET AL., 1975; PRESTON & STOCKLEY, 2006; RAMM & STOCKLEY, 2014; CHAPTER 1), we also recorded 6) the total number of ejaculations

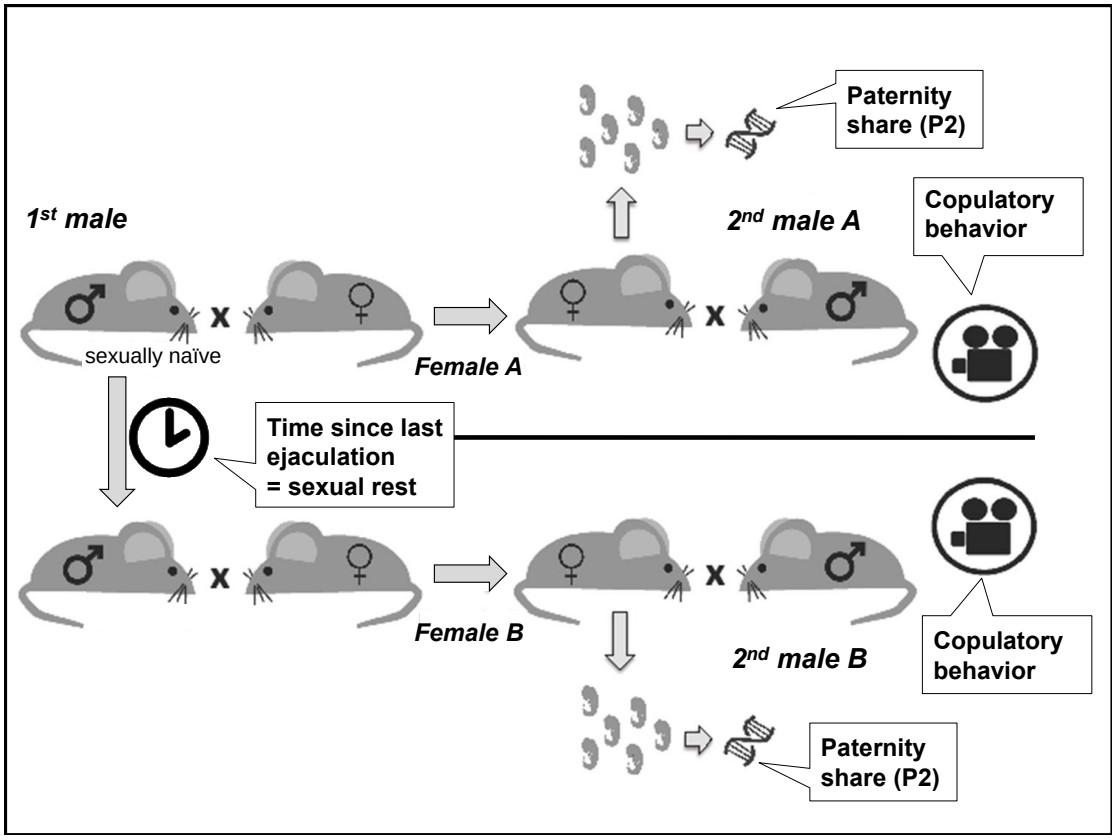

Figure 4.1

Experimental design of the second experiment. A sexually naive first male was mated to a receptive female A, which was subsequently paired with second male A. The first male was then paired with another receptive female B. After ejaculation, female B was paired with second male B. The copulatory behaviour of both second males was remotely recorded. Females were sacrificed 12–14 days *post coitum*, and paternity of the embryos was determined using 12 microsatellite markers. We analysed copulatory behaviour and P_2 as a function of sexual restedness of the first male.

4.3.5 Paternity share

Only 19 of the 52 females were pregnant 12–14 days *post coitum*. Tissue samples were taken *post mortem* from all embryos, their mothers, and their potential sires. DNA was extracted using the EDNA HISPEX extraction kit (Fisher Biotec, Subiaco, Western Australia, Australia). For paternity assignment, we scored 12 microsatellites spread across 10 autosomes (D3Mit278, D4Mit227, D5Mit122, D5Mit352, D6Mit139, D6Mit390, Chr8_3, D10Mit230, D11Mit90, D14Mit44, D16Mit139, and Chr19_17). Marker and polymerase chain reaction details are described elsewhere (BULT ET AL., 2008; TESCHKE ET AL., 2008; LINDHOLM ET AL., 2013). Paternity analysis using the known mother and the 2 candidate fathers was performed using the software CERVUS (KALINOWSKI ET AL., 2007) and a genotyping error rate of 0.01 (LINDHOLM ET AL., 2013). Paternity assignments were accepted at a confidence level of 95% with a single or no mismatch between offspring and assigned father.

4.3.6 Statistical analyses

All statistical analyses were performed in R, version 3.1.0 (R Core Team, 2015). In the first experiment, we explored variation in plug size after repeated ejaculation and variable sexual restedness. We assumed that replenishment of the seminal vesicles that produce the majority of constituents of the copulatory plug would follow an asymptotic function. We analysed differences between first and second plugs as a function of time difference between a male's 2 ejaculations using a 3-parameter asymptotic function with the asymptote of the difference between 2 consecutive plugs fixed to 0 (full replenishment over time). Thus, we estimated only 2 of the 3 parameters using the nls function in R: the response when time delay is 0 and the rate constant of the asymptotic growth (see Wilson et al. 2014). We compared the asymptotic model against a null model where plug size remains constant over time (i.e., intercept model) based on the Akaike information criterion corrected for small sample sizes (AICc).

In the second experiment, we investigated whether sexual restedness of first males affected the copulatory behaviour and paternity success of second males. As a predictor variable, we used variation in sexual restedness of the first male, measured as time since his last ejaculation. However, our males were initially sexually inexperienced so that restedness was maximal and could not be quantified as time rested. Based on the trajectory of plug size differences from the first experiment and on sperm replenishment in a recent experiment using these house mouse populations (Firman et al., 2015b), we assumed that copulatory plug fluid reserves would be fully replenished after a week and assigned the maximum value of 7 days sexual restedness to sexually naive males and to males rested for more than a week.

Copulatory behavioural traits of second males were correlated and therefore were reduced using a Principal component analysis (PCA). We transformed variables to approach normality using log(x + 1) transformation, with the exception of *the number of copulatory bouts*, which was transformed using sqrt(x + 1). We tested for an effect of sexual restedness of the first male (applied here as a proxy for plug size) on the copulatory behaviour of second males with linear mixed models (LMMs), using the function lmer implemented in lme4 (Bates et al., 2014). Males that did not mount the female ($n = 11$) and that did not ejaculate despite mounting ($n = 8$) could not be included in the PCA due to missing data. For these males, we analysed the occurrence of mounting and of ejaculation by the second male with binary generalized LMMs (GLMMs) using the function glmer in the package lme4 (Bates et al., 2014), including time since previous ejaculation of the first male as a fixed effect and the identity of the first male as a random effect to account for our paired design. Copulatory behaviour is likely influenced by a range of parameters, and using significance thresholds to remove predictor variables can lead to biased estimates (Forstmeier & Schielzeth, 2011). We thus used an information-theoretic approach to incorporate uncertainty in parameter estimates as well as in model selection while retaining our focus on the effect of the copulatory plug. We fitted full models including either the first or the second principal component of copulatory behaviour as the dependent variable, time since previous ejaculation of the first male, the second male's body weight, and population origin as fixed effects. To account for our paired design and to avoid pseudoreplication, the identity of the first male was included as a random effect. We followed the recommendations of Grueber et al. (2011) for model averaging based on AICc. Using the dredge function in the MuMIn package (Bartoń, 2015), we ran a full submodel set and selected all models within a range of 4 AICc units and averaged across models, using Akaike weights. Because of our interest in the effect of sexual restedness of the first male, we used the natural average method (Grueber et al., 2011).

We analysed paternity share of the second male (P_2) with GLMMs, using the function glmer. The number of embryos sired by the second male was included as the dependent variable and the number of offspring genotyped as the binomial denominator. Paternity outcome is likely determined by a complex interaction of different effects. However, due to the small sample size for paternity share caused by pregnancy failure, we fitted simple models that included only a few covariates to avoid model overfitting. In the full model, time since previous ejaculation of the first male, and the 2 first principal components for copulatory behaviour of the second male were included as fixed effects. To avoid pseudoreplication, we included identity of the first male as a random factor. Similar to the analyses on copulatory behaviour, we ran a full submodel set and selected models within 4 AICc units for natural averaging (Grueber et al., 2011). Dispersion parameters of the GLMMs were < 1. Means $\pm$ SE are presented.

4.3.7 Ethical statement

This research was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the UWA Animal Ethics Committee (approval number: RA/3/100/1306).

4.4 Results

4.4.1 Variation in plug size across successive copulations

In the first experiment, we investigated plug weights when males had ejaculated twice, between two and 56 h apart ($n = 27$). Three males produced a plug but failed to ejaculate a second time within three days, and so were only included in the analyses of first plugs. The weight of first plugs was significantly associated with male body weight, but relative plug size did not differ according to source population (ANOVA: body weight $F_{1,26} = 5.62$, $P = 0.026$; population origin $F_{2,26} = 1.05$, $P = 0.334$). Populations differed in the time difference between two ejaculations, with Rat Island males being most likely to ejaculate twice on the same day (Rat Island 8/10, Boullanger Island 3/8, Whitlock Island 2/9; $\chi^2 = 6.85$, df $= 2$, $P = 0.033$). First plugs were larger than second plugs (first plugs 44.5 $\pm$ 3.3mg, second plugs 25.3 $\pm$ 2.3mg; paired t-test, $t_{27} = 5.66$, $P < 0.001$), and the difference between first and second plug weight tended to decrease with increasing time between the two ejaculations (Figure 4.2), although the asymptotic model obtained only a marginally better AICc support than the null model (asymptotic model: AICc = 229.7, intercept model: AICc = 229.9). Time since last ejaculation only explained a small proportion of the variation in plug size differences (quasi-$R^2 = 0.1$). As such, time since last ejaculation was a weak predictor for the size of the second plug. When we omitted males that had produced two plugs during the same dark cycle (up to 7 h time difference), there was a smaller but still significant difference in plug size (mean difference 11.1 $\pm$ 3.9 mg; paired t-test, $t_{13} = 2.88$, $P = 0.013$).

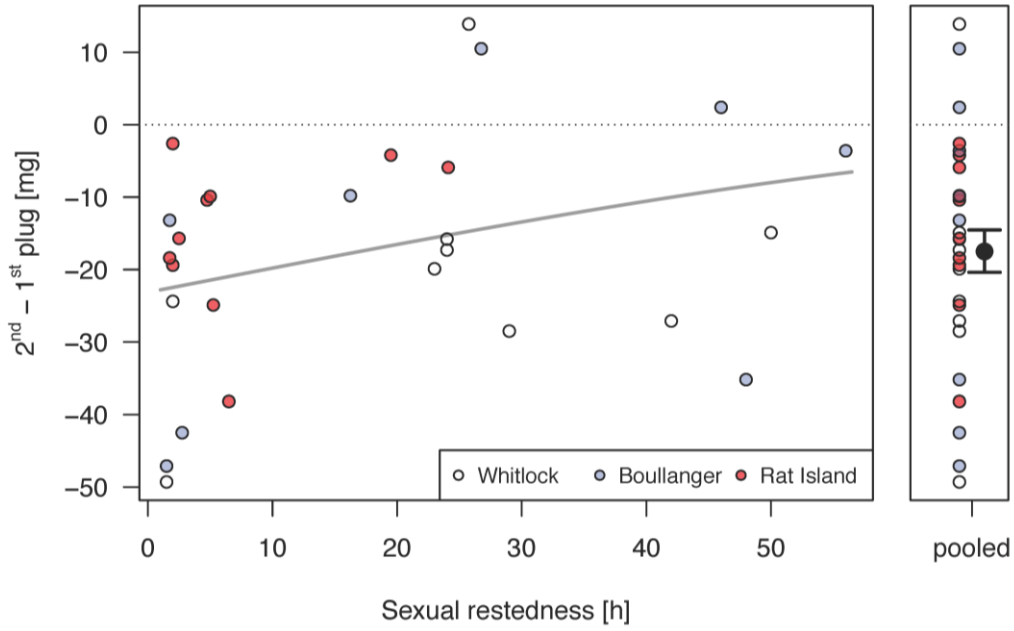

Figure 4.2

Differences in plug weights between males' first and second plugs in experiment 1. Plug weight differences (milligram) are shown as a function of time difference between a male's 2 ejaculations (sexual restedness). Point color indicates the population the mice were derived from, with white through blue to red increasing with multiple paternity levels (FIRMAN & SIMMONS, 2008B). The gray line indicates the model prediction from a 3-parameter asymptotic model (see main text). A pooled version of all differences and the overall mean difference ± SE is shown in the right panel.

4.4.2 First male sexual restedness and second male copulatory behaviour

In the second experiment, we used two consecutive ejaculations of first males to investigate the effect of male mating status, and consequently plug size, on the copulatory behaviour of second males to mate. Fifty-two females mated with a first male and were subsequently paired with a second male. In 79% of the trials, the second male attempted to mate with the female, as evidenced by at least one mount. Eleven trials were omitted from further analyses because we could not ascertain that the female was still sexually receptive as evidenced by mounting. There was no effect of time since previous ejaculation of the first male on the probability of mounting by the second male (GLMM: 52 trials, 27 first males, $z = 0.74$, $P = 0.457$, b [95% confidence interval CI] = 0.09 [-0.16, 0.35]). We then omitted trials in which the second male mounted the female but did not ejaculate (8/41 trials). The probability of ejaculation by the second male was not influenced by time since previous ejaculation of the first male (GLMM: 41 trials, 26 first males, $z = -0.70$, $P = 0.485$, b [95% CI] = -0.39 [-1.53, 0.75]).

The PCA on copulatory behaviour of males copulating to ejaculation yielded two principal components with eigenvalues larger than 1. The first component (PC1) explained 46% of the variation in copulatory behaviour. PC1 was negatively loaded by the number of mounts/intromissions and ejaculation latency, and positively loaded by the number of

ejaculations (Table 4.1). The second component (PC2) explained 21% of the variation and was positively loaded by mount latency and negatively loaded by intromission latency. Given the positive loading of the number of ejaculations and the negative loading of latency to first ejaculation, PC1 can be interpreted as ejaculatory ease, with males obtaining high PC1 values reaching ejaculation sooner and more often than males with low PC1 values. For PC2, long latencies to the first mount coincided with short latencies to the first mount with intromission. PC2 can thus be interpreted as copulatory delay, with higher scores indicating a long latency to the onset of copulation. We used PC1 and PC2 for further analyses. Model selection and effect sizes from model averaging indicated that ejaculatory ease of the second male (PC1) tended to decrease with sexual restedness of the first male (Figure 4.3). The model including only sexual restedness obtained the best AICc support although the null model obtained similar support (ΔAICc = 0.72; Table 4.2). The effect size of sexual restedness on ejaculatory ease was negative. However, the 95% CI overlapped 0 (b [95% CI] = -0.64 [-1.34, 0.06]). Variation in PC2 was most strongly influenced by body weight of the second male to mate, with heavier males showing shorter copulatory delay (standardized effect size b [95% CI] = -1.10 [-1.86, -0.34]). Sexual restedness of the first male did not have an effect on PC2 (b [95% CI] = 0.10 [-0.61, 0.82]).

Table 4.1

Behavioral trait	*Mean*	*SD*	*PC1*	*PC2*
Time of first mount (mount latency) [s]†	1100	1268	0.316	**0.656**
Latency to first intromission [s]†	280	299	-0.379	**-0.702**
Number of copulatory bouts‡	29	19	**-0.883**	-0.175
Latency to ejaculation [s]†	1833	996	**-0.912**	0.156
In copula duration at first ejaculation [s]†	11.4	4.4	0.520	-0.438
Number of ejaculations	1.2	0.4	**0.795**	-0.324
Eigenvalue	-	-	2.76	1.28
% explained	-	-	46.6%	21.3%

† log(x+1) transformed for PCA; ‡ sqrt(x+1) transformed for PCA

Observed copulatory behavioural traits, their variability indices and results from a principal component analysis (PCA). Eigenvectors in bold were interpreted as contributing significantly to the PC.

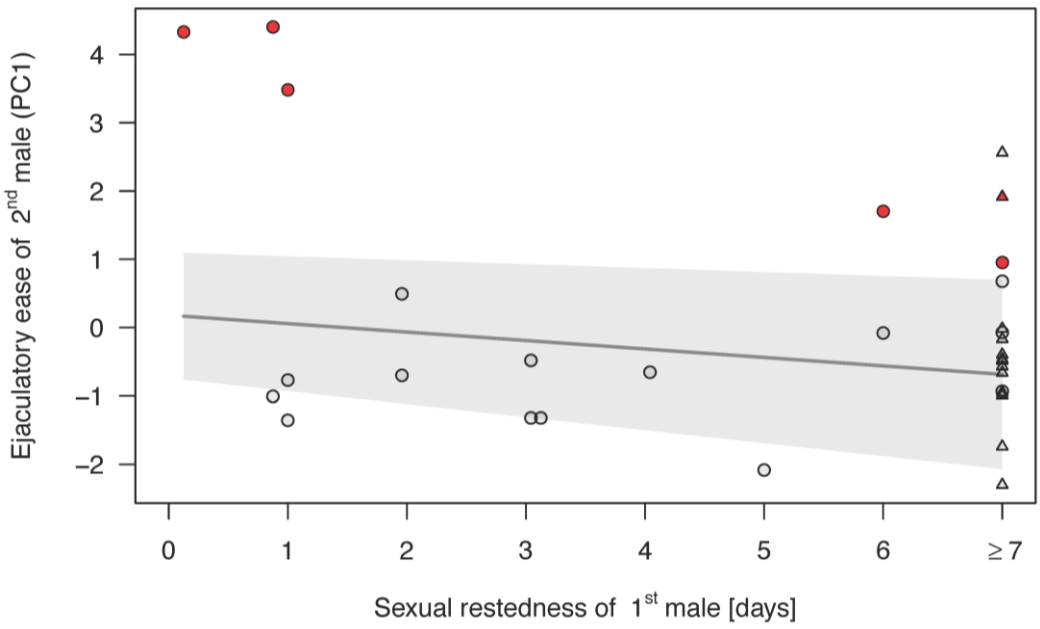

Figure 4.3

Ejaculatory ease (PC1 of copulatory behaviour) of second males to mate as a function of sexual restedness of the first male. Males that did not ejaculate were omitted for the PCA, and males that ejaculated twice are indicated in dark red. Males rested for longer than 7 days were assumed to be fully rested and were pooled. Sexual restedness of sexually naive first males (triangles) is maximal. For the analyses, we assigned a maximal value of 7 days. The line and shaded area indicate model predictions of the mean effect of sexual restedness ± SEM with body weight and population origin centred. The effect size and unconditional SE were obtained from model averaging of LMMs. Ejaculatory ease tended to be higher when sexual restedness was short (see main text). SEM, standard error of the mean.

Table 4.2

	Intercept	Sexual rest 1st male	Body weight 2nd male	Population: Rat	Whitlock	df	AICc	ΔAICc	*w*
Model 1	0.02	-0.64				4	119.2	0	0.39
Model 2	0.01					3	119.9	0.72	0.27
Model 3	0.02	-0.65	0.08			5	122	2.8	0.10
Model 4	0.00		0.08			4	122.5	3.32	0.07
Model 5	-0.90	-0.61			+	6	122.6	3.49	0.07
Model 6	-1.03				+	5	122.7	3.54	0.07
Model 7	-1.04		0.04		+	6	125.7	6.58	0.02
Model 8	-0.91	-0.62	0.02		+	7	125.9	6.79	0.01
Estimate	-0.17	-0.64	0.08	1.02	1.81				
Unconditional SE	0.61	0.34	0.51	0.90	1.11				
Lower 95% CI	-1.40	-1.34	-0.97	-0.82	-0.48				
Upper 95% CI	1.06	0.06	1.13	2.87	4.10				
Relative importance		0.57	0.18	0.14					

Random terms: 1|male1

df = degrees of freedom; *w* = relative model weights

Model summary statistics of submodels on ejaculatory ease. The full model included sexual restedness of the first male, body weight of the second male and population origin as fixed effects, and the identity of the first male as a random effect. Models within four AICc units of the best model were used for estimating standardized effect sizes using the natural average (highlighted in bold).

4.4.3 First male sexual restedness and second male paternity share

Of 52 females that received an ejaculation by at least 1 male, only 19 had implanted embryos at the time of dissection. Pregnancy was not associated with female body weight at the time of mating (GLMM: 52 trials, 27 first males, $z = 0.53$, $P = 0.596$, b [95% CI] = 0.31 [-0.86, 1.48]), sexual rest of the first male (z = -0.09, P = 0.929, b [95% CI] = -0.05 [-1.22, 1.11]), or with whether the second male ejaculated ($z = 1.15$, $P = 0.250$, b [95% CI] = 0.72 [-0.54, 1.99]). Of the 19 pregnant females, we excluded 5 from trials during which the second male had not ejaculated. Thus, our final sample size for paternity share analyses was 14 trials where both males had ejaculated. The corresponding number of implanted embryos was 99 (mean per female = 7.1, range 5–9), of which 8 embryos (8%) could not be assigned a father. The rate of multiple paternity was 57%, with six females having all embryos sired by a single male (in four cases by the first male). Second males sired a smaller proportion of offspring than first males (mean P_2: 0.33 ± 0.09), in agreement with a first male advantage previously described for house mice (Firman & Simmons, 2008a). In a univariate analysis, sexual restedness of the first male had a significant negative effect on P_2 (GLMM: 14 trials, 11 first males, z = -2.52, P = 0.012, b [95% CI] = -1.96 [-3.65, -0.28]), showing that first males that had recently mated had a lower paternity share than first males that had not mated recently. After incorporating additional variables, model comparison revealed that the model with the lowest AICc value included sexual restedness of the first male and ejaculatory ease (PC1) of the second male, but a model including only ejaculatory ease obtained an AICc value that was only 1.5 units larger (Table 4.3). Effect sizes after model averaging indicated that ejaculatory ease had a strong positive effect on P_2 (b [95% CI] = 3.86 [1.55, 6.17]), whereas sexual restedness of the first male had a negative but non-significant effect

on P_2 (b [95% CI] = −1.67 [−3.33, 0.01]; Figure 4.4). Sexual restedness and ejaculatory ease showed only weak collinearity (variance inflation factors < 1.3).

Table 4.3

	Intercept	Sexual rest 1st male	Ejaculatory ease [PC1]	Copulatory delay [PC2]	df	AICc	ΔAICc	w
Model 1	-0.56	-1.66	4.02	–	4	41.1	0	0.60
Model 2	-0.79	–	3.52	–	3	42.6	1.51	0.28
Model 3	-0.55	-1.64	3.79	-0.47	5	45.4	4.34	0.07
Model 4	-0.77	–	3.39	-0.47	4	45.9	4.87	0.05
Model 5	-1.12	-1.96	–	–	3	55.8	14.78	<0.01
Model 6	-1.32	–	–	–	2	59.2	18.13	<0.01
Model 7	-1.06	-2.06	–	-0.47	4	59.6	18.54	<0.01
Model 8	-1.47	–	–	0.72	3	62	20.97	<0.01
Estimate	-0.63	-1.67	3.86					
Unconditional SE	0.31	0.75	1.04					
Lower 95% CI	-1.32	-3.33	1.55					
Upper 95% CI	0.06	0.01	6.17					
Relative importance		0.68	1					

Random terms: 1|male1

Model summary statistics of submodels on P_2. The full model included sexual restedness of the first male and both principal components of copulatory behaviour of the second male as fixed effects, and the identity of the first male as a random effect. Models within four AICc units of the best model were used for estimating standardized effect sizes using the natural average (highlighted in bold).
df = degrees of freedom; w = relative model weights

4.5 Discussion

Copulatory plugs are deposited by males at mating in a large variety of taxa and have been posited to be an adaptation to postcopulatory competition, providing fitness benefits through the avoidance of or engagement in sperm competition. Here, we show that male house mice produced smaller plugs when ejaculating after a shorter period of sexual rest, and thus appear to be significantly limited in producing seminal fluids that result in plug formation. We assume that sexually rested males may also have been able to produce ejaculates containing more sperm. We found only weak support for the hypothesis that plugs represent a physical barrier to sperm competition rivals. Although larger plugs tended to be associated with later ejaculation by second males, this effect was not statistically significant. Males in the second-to-mate role obtained a lower paternity share when competing against sexually rested males, which were able to produce a large plug. This is possibly due to effects of the plug on both ejaculation latency and sperm retention. Our experimental design did not allow us to disentangle the effects of plug size and ejaculate size, but a reduction in plug size may accentuate a reduction in ejaculate size, if large plugs promote sperm retention in the female reproductive tract.

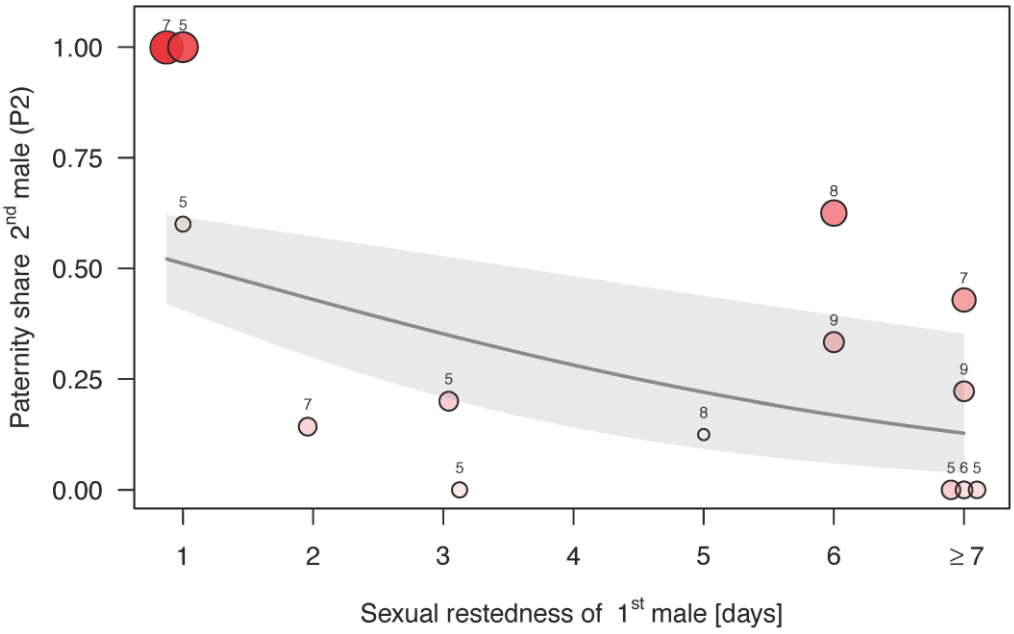

Figure 4.4

P_2 as a function of restedness of their competitor. Point size and redness are proportional to PC1 scores. Numbers indicate the number of embryos genotyped. The line and shaded area indicate model predictions of the mean effect of sexual restedness ± SEM for an average PC1 score ± SEM. The effect size and unconditional SE were obtained from model averaging of GLMMs and back transformed using the inverse logit. Restedness of the first male to mate tended to negatively affect P_2, and ejaculatory ease of the second male to mate had a strong positive effect on P_2 (see main text). SEM, standard error of the mean.

4.5.1 Constraints on plug production

When males ejaculated twice over a period of a few days, the copulatory plug they deposited was smaller at the second ejaculation. We did not experimentally manipulate the time difference between two ejaculations but attempted to get second ejaculations as soon as possible and opportunistically explored the resulting variation. Although a large proportion of males used in this experiment ejaculated twice on the same day (13/30 = 43%), some males had a longer time difference between their 2 ejaculations and for three males we did not obtain two plugs within three days. The time difference between the two ejaculations was associated with the level of sperm competition in the populations from which the mice were originally derived (Firman & Simmons, 2008b). Males from the population with the most intense sperm competition (Rat Island) exhibited the shortest time difference between two ejaculations. It is plausible that the high level of sperm competition on Rat Island has selected for a higher mating potential in these males (Linklater et al., 2007). In accordance with sperm competition theory, Rat Island population males have also been found to produce greater numbers of sperm compared with males from the other two populations (Firman et al. 2013, 2015). However, we cannot rule out that the observed pattern

was due to other factors, such as differences in female oestrous length, receptivity, or in our ability to detect receptivity based on vaginal appearance (Byers et al., 2012) among these populations.

First plugs were positively correlated with male body weight, but relative plug weight did not differ between mice from populations with different histories of sperm competition intensity. This is in agreement with previous reports that sperm competition cues in the social environment or in the immediate mating context do not influence plug size (Ramm & Stockley, 2007; Klemme & Firman, 2013). The size difference between two consecutively produced plugs tended to decrease over time, indicating the need for seminal fluid replenishment between matings. Thus, when males ejaculated twice on the same day, the plug produced at their second ejaculation was reduced in size on average by 50% (-24 mg), but one or two days later this reduction in plug size was only 19% (-11 mg). There was large among male variation in the difference in size between first and second plugs, which we could not explain. Given the low sample size, large individual variation and the limited variation in the time difference between two ejaculations, our data do not fully support recovery of plug size over time. However, our data show that males are significantly plug limited after a recent ejaculation, and full recovery likely takes place in sexually mature males when given sufficient time. Thus, even though our findings do not allow an estimation of the rate of recovery, our results suggest that full recovery of a male's plug-producing capacity may take up to three days and that males are significantly plug limited after a recent ejaculation. These findings enabled us to use time since last ejaculation as a broad proxy for plug size in exploring plug function.

4.5.2 Is the plug a barrier to copulations by rival males?

In our second experiment, we investigated how variation in plug size, as estimated by the duration of sexual rest among first males, affected the copulatory behavior of a second male and his paternity outcome. We found no evidence for an association between the extent to which a first male had been sexually rested and the second male's sexual interest or likelihood of ejaculation. However, experimental difficulties with reducing the length of sexual restedness of first males call for prudence in interpreting these results. Only 16/27 (59%) first males copulated with two different females within three days, out of which only two ejaculated twice on the same day. Our data from the first experiment showed that plug size reduction was substantial when males were rested for less than a day and that plug size was largely restored after this time. Thus, average plug size differences between sexually naive and variably sexually rested males might have been too small to represent large differences in terms of physical resistance that would affect sexual interest or ejaculation likelihood.

Overall, the rate of female remating was high and was not influenced by the sexual restedness of first males (33/41 second males ejaculated). This is in agreement with other laboratory studies in house mice that found evidence for high rates of multiple mating without experimental plug removal (20/21 in Rolland et al. 2003; at least 57/78 in Chapter 1). Moreover, as found here and in previous studies (Estep et al., 1975; Preston & Stockley, 2006; Ramm & Stockley, 2014; Chapter 1), males occasionally ejaculate more than once with the same female, supposedly removing their previously deposited copulatory plug before their second ejaculation. This provides further indications that the plug does not prevent subsequent copulations. Nevertheless, a plug could benefit its producer by delaying ejaculation by competitor males and enhancing the first male's paternity share. Ramm and Stockley (2014) found that males preferred to mate with unmated females compared with recently mated females, as evidenced by a lower mating success with

mated females. Copulating with mated females involved more intromissions and a longer ejaculation latency, potentially due to resistance imposed by the copulatory plug, and thus might be energetically more costly than copulating with unmated females (Ramm & Stockley, 2014). To look at the effects of plug size variation on copulatory behaviour, we reduced variation in the observed behaviours of second males that had achieved ejaculation to two main principal components: ejaculatory ease and copulatory delay. If the copulatory plug represented an effective mechanical barrier to copulation and larger plugs provided higher effectiveness, one might predict a negative effect of first male sexual restedness (i.e., larger plugs) on ejaculatory ease of the second male. Indeed, the negative effect size of sexual restedness of the first male on ejaculatory ease of the second male aligns with the prediction that larger copulatory plugs lead to a longer ejaculatory delay, but the CIs of the effect were broad and overlapped 0. Given the aforementioned limitations of our experimental approach, our estimate of the effect of plug size on rival behaviour was associated with substantial uncertainty. The size of mouse copulatory plugs does not appear to be adjusted in response to the perceived risk of sperm competition (Ramm & Stockley, 2007; Klemme & Firman, 2013), despite males responding to the immediate risk of sperm competition in other copulatory features (Preston & Stockley, 2006; Ramm & Stockley, 2007). Moreover, males respond to sperm competition cues in their social environment by increasing sperm production (Firman et al., 2013), but not seminal vesicle size (Ramm & Stockley, 2009). Collectively, these findings do not support the hypothesis that the house mouse plug serves a significant function in preventing female remating but may nonetheless represent a physical obstacle for rival males to overcome. Notably, a recent study found that after monogamous matings, small plugs persisted in the female reproductive tract for longer than large plugs despite being more susceptible to proteolytic degredation by females (Mangels et al., 2015). The authors suggested that smaller plugs may be more difficult to remove by females, whereas large plugs may be more difficult to remove by competitor males (Mangels et al., 2015), and our study lends some support to the latter hypothesis.

4.5.3 Does the plug influence paternity outcome?

We found that paternity share of second males (P_2) decreased as the time since previous ejaculation of the first male increased. Higher ejaculatory ease of second males, which tended to be associated with short sexual restedness of first males, had a strong positive effect on P_2. Notably, after controlling for the effect of ejaculatory ease of the second male, sexual restedness of the first male still tended to influence P_2, although the 95% CI overlapped 0. The number of ejaculated sperm is a major determinant of paternity success in sperm competition in mammals (Gomendio et al., 1998). Meadow voles respond to an elevated risk of perceived sperm competition through ejaculation of larger sperm numbers without altering ejaculation frequency (DelBarco-Trillo & Ferkin, 2004) whereas male house mice have been shown to respond through multiple ejaculations (Preston & Stockley, 2006) and increased sperm production (Ramm & Stockley, 2009; Firman et al., 2013). Meta-analyses across animal taxa have shown that males respond to an increased risk of sperm competition by allocating more sperm (DelBarco-Trillo, 2011; Kelly & Jennions, 2011). Our results confirm that repeated ejaculation can confer a fitness benefit through an increase in paternity share because PC1 (ejaculatory ease) had a strong effect on paternity share and was loaded strongly by the number of ejaculations. However, because of collinearity between the latency to ejaculation and the number of ejaculations, we cannot disentangle the effects of the number of ejaculations and the delay between the two rivals' ejaculations. Likewise, the effect of the first male's sexual restedness on paternity share might be attributable to the number of the first males'

sperm in competition because there was still a trend after controlling for variation in the second male's ejaculation latency and number of ejaculations. Little is known about ejaculate size as a function of time since last ejaculation in mice, but full sperm replenishment in male rodents typically takes up to a week (Ramm and Stockley 2014 and references therein). In humans, ejaculate size increases as a function of time since last ejaculation for at least one week (Baker & Bellis, 1993). It is thus plausible that our observed negative effect of first male sexual restedness on P_2 was caused entirely by slow recovery in the number of sperm ejaculated. Interestingly however, in a recent experiment performed on mice from these populations, the number of epididymal sperm did not significantly differ among males that had been sexually rested for two months and males that had mated between three and five days prior, although the direction of the effect is consistent with sperm depletion (Firman et al., 2015b). Alternatively, a reduction in plug size accompanied by sperm limitation may contribute to the observed sperm competition outcome through decreased sperm retention (Parker, 1970). When males ejaculated twice on the same day, uterine sperm numbers were reduced even more drastically (by 80%; Huber et al. 1980) than the copulatory plug in our study (~50% reduction). If small copulatory plugs are deficient in assisting sperm transport into the uterus (Carballada & Esponda, 1992; Dean, 2013), a reduction in plug size could interact with an underlying decrease in the number of sperm ejaculated, exacerbating the reduction in uterine sperm numbers. Thus, large copulatory plugs could be beneficial in sperm competition by ensuring optimal sperm transfer (Ramm & Stockley, 2007).

Unfortunately, a substantial proportion of mated females did not become pregnant, greatly reducing the sample size for our paternity analysis. Pregnancy failure was not related to female body weight or sexual rest of the first male, but could be related to the relatively young age of females and their lack of reproductive experience. Alternatively, pregnancy failure could be related to the Bruce effect, the block of pregnancy by exposure of mated females to a non-stud male or his odour (Bruce, 1959). However, we did not find the association between female remating and pregnancy (i.e., pregnancy block by females that did not remate) predicted by the Bruce effect. Other studies that used a similar competitive mating design did not find high rates of pregnancy failure, suggesting that exposure to more than 1 male per se does not lead to pregnancy failure (Firman & Simmons, 2008a; Chapter 1). Because of the small sample size, we focused on variables that were at the centre of interest of our study (first male sexual restedness and second male copulatory behaviour).

4.5.4 Evolutionary implications

Fromhage (2012) modelled the maintenance of plug efficiency under varying levels of female remating, and found that high rates of polyandry are expected to result in low plug size and efficiency, because as males get mating opportunities, they invest more heavily into sperm production and mating capacity rather than into copulatory plugs. The model assumed that copulatory plugs only affected the likelihood of female remating. Our study supports the notion that a decrease in plug size might also affect the outcome of sperm competition through delaying remating or/and influencing sperm transport. This might provide an evolutionary incentive for large plugs arising from sperm competition even if they are relatively ineffective at preventing female remating (Parker, 1970).

However, differences between taxa are likely to be important in determining the costs and benefits of copulatory plugs, limiting the generality of our findings. Even among rodents, there are indications for differential plug effectiveness. Although the plug was found to be an effective

mate guard in guinea pigs (Martan & Shepherd, 1976), there was no effect of experimental plug removal on the paternity outcome in deer mice (Dewsbury, 1988a). Bank voles increase the size of their seminal vesicles in response to social cues to sperm competition but do not increase sperm production (Lemaître et al., 2011), whereas the inverse pattern was found in house mice (Ramm & Stockley, 2009). The effectiveness and maintenance of copulatory plugs as a mating block may be greatly determined by the reproductive biology of the species being considered. For example, costs and benefits of plugging females may depend on the operational sex ratio, sexual size dimorphism, length of female receptivity, level of polyandry, sperm and seminal fluid depletion rates, sperm precedence patterns, and plug removal skills (Dunham & Rudolf, 2009; Fromhage, 2012). Copulatory plugs may also be subject to sexual conflict over female remating (Koprowski, 1992; Stockley, 1997; Mangels et al., 2015), which could lead to coevolutionary dynamics between male manipulation and female control over plug efficacy and thus to different levels of plug efficacy among different species that are evolving under very similar selective forces. Currently available data on house mice suggest that the dynamics of copulatory plugs are complex (Mangels et al., 2015), that plugs may be necessary for fertility (Dean, 2013), and that large plugs may provide fitness benefits to males when engaging in sperm competition.

4.5.5 Concluding remarks

Using controlled experimental matings, we show that after a single ejaculation male house mice became limited in the seminal fluids that produce the plug and recover relatively slowly. Although the effect was not significant, the size of a first-to-mate male's copulatory plug tended to delay ejaculation of a second-to-mate rival male. First males that had recently mated obtained a smaller paternity share in sperm competition relative to first males that had been rested. This was probably due to a combination of both small plug and small ejaculate production, resulting in a shorter ejaculation delay for rival males and in fewer sperm being transported to the fertilization site, respectively. Thus, current evidence in house mice suggests that the copulatory plug does not represent a strong barrier to copulation, but might still offer an advantage in sperm competition by delaying remating and ensuring efficient sperm transport.

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CHAPTER 5 THE COPULATORY PLUG DELAYS EJACULATION BY RIVAL MALES AND AFFECTS SPERM COMPETITION OUTCOME IN HOUSE MICE

5.1 ABSTRACT

Females of many species mate with multiple males (polyandry), resulting in male–male competition extending to post-copulation (sperm competition). Males adapt to such post-copulatory sexual selection by altering features of their ejaculate that increase its competitiveness and/or by decreasing the risk of sperm competition through female manipulation or interference with rival male behaviour. At ejaculation, males of many species deposit copulatory plugs, which are commonly interpreted as a male adaptation to post-copulatory competition and are thought to reduce or delay female remating. Here, we used a vertebrate model species, the house mouse, to study the consequences of copulatory plugs for post-copulatory competition. We experimentally manipulated plugs after a female's first mating and investigated the consequences for rival male behaviour and paternity outcome. We found that even intact copulatory plugs were ineffective at preventing female remating, but that plugs influenced the rival male copulatory behaviour. Rivals facing intact copulatory plugs performed more but shorter copulations and ejaculated later than when the plug had been fully or partially removed. This suggests that the copulatory plug represents a considerable physical barrier to rival males. The paternity share of first males increased with a longer delay between the first and second males' ejaculations, indicative of fitness consequences of copulatory plugs. However, when males provided little copulatory stimulation, the incidence of pregnancy failure increased, representing a potential benefit of intense and repeated copulation besides plug removal. We discuss the potential mechanisms of how plugs influence sperm competition outcome and consequences for male copulatory behaviour.

Key words: Copulatory plug, sperm competition, copulatory behaviour, polyandry, house mouse

5.2 Introduction

Females of many species mate with multiple males (polyandry), leading to post-copulatory competition between males (Parker, 1970). Males are predicted to respond to this strong evolutionary force through adaptations in ejaculate production and allocation (Simmons, 2001; Wedell et al., 2002). Males may also maximize their fitness by manipulating or guarding females (Parker, 1970; Gillott, 2003). Copulatory plugs that obstruct the female genital tract and are secreted from males at ejaculation have evolved independently in many different taxa (e.g. insects (Orr and Rutowski 1991) and primates (Dixson and Anderson 2002)), presumably to prevent subsequent inseminations by rival males (Parker, 1970). A role for copulatory plugs in postcopulatory competition has been inferred indirectly in comparative studies on butterflies (Simmons, 2001), spiders (Uhl et al., 2010), rodents (Ramm et al., 2005) and primates (Dixson, 1998). Moreover, relative testis size in rodents (Ramm et al., 2009) and polyandry levels in primates (Dorus et al., 2004) show positive associations with evolutionary rates of coagulating semen components, supporting a role for copulatory plugs in post-copulatory competition. Direct experimental evidence is, however, mixed. A variety of studies have found an effect of the plug on the outcome of sperm competition (e.g., Masumoto, 1993; Shine et al., 2000; Polak et al., 2001; Kunz et al., 2014), while others have not (e.g., Moreira and Birkhead 2003; Timmermeyer et al. 2010). Given that females benefit from multiple mating in many species (Jennions & Petrie, 2000), they may counteract male attempts to prevent remating (Koprowski, 1992; Stockley, 1997; Friesen et al., 2016),), leading to sexual conflict over plug efficacy and co-evolutionary dynamics between both males and females as well as between rival males in plugging and plug removal efficacy (Fromhage, 2012). Thus, even if copulatory plugs are relevant to post-copulatory competition, these evolutionary conflicts over plug efficacy between the sexes and between rivals are likely to lead to situations in which copulatory plugs are not fully effective in preventing female remating.

For rodents, the role of post-copulatory competition in the evolution of copulatory plugs is unclear (Ramm & Stockley, 2016), and a number of other nonmutually exclusive functions of copulatory plugs have been hypothesized, such as gradual sperm release, sperm transport and cervical stimulation (reviewed in Voss, 1979). Comparatively, rodent species with relatively larger testes, a proxy for sperm competition intensity, have relatively larger seminal vesicles – the organs responsible for producing plug proteins – and relatively larger copulatory plugs (Ramm et al., 2005). Within species, a significant effect of experimental plug removal on female remating in the guinea pig (Martan & Shepherd, 1976) contrasts with no effect of experimental plug removal on paternity outcome in deer mice (Dewsbury, 1988a). In house mice, males produce large copulatory plugs from coagulating proteins that are secreted from both the seminal vesicles and the coagulating glands and that comprise about one-third of all semen proteins (Dean et al., 2011). Copulatory plugs may be important for pregnancy initiation by temporally extending vaginal stimulation beyond the ejaculatory reflex (McGill & Coughlin, 1970; Leckie et al., 1973). Males that lack the transglutaminase IV gene and cannot form a copulatory plug show reduced fertility, probably because of dramatically reduced sperm transport through the female reproductive tract (Dean, 2013). Thus, aiding sperm transport may be another potential function of the plug. Yet, when females are isolated after mating, plugs remain in the female reproductive tract for a prolonged

period of time (49% of plugs still present after 24 h; Mangels et al. 2015), longer than behavioural oestrus and the fertilization life of the ovum (Table I in Voss, 1979). Also, plug removal does not reduce pregnancy rates (Firman & Simmons, 2010), unless removed before coagulation (Bloch, 1972). Why male mice produce such large and long-lasting plugs might only be understood when considering post-copulatory competition between males. Even if the copulatory plug evolved under selective forces associated with the effective sperm transport or pregnancy initiation, it is plausible that the copulatory plug has subsequently evolved to fulfil additional functions related to post-copulatory competition.

Multiple paternity is common in natural house mouse populations (Dean et al., 2006; Firman & Simmons, 2008b; Lindholm et al., 2013), and females mate multiply in the laboratory when given a free choice (Rolland et al., 2003; Manser et al., 2015). Some studies have directly observed female remating after the deposition of a copulatory plug, showing that copulatory plugs do not prevent remating (Ramm & Stockley, 2014; Chapters 1 & 4). Nonetheless, large copulatory plugs could be beneficial in the context of sperm competition by delaying ejaculation of rival males to a suboptimal time relative to ovulation (Parker, 1970; Chapter 4). In house mice, first males sire the majority of offspring even if plugs are removed (Levine, 1967; Firman & Simmons, 2008a), probably because they ejaculate at an optimal time relative to the release of ova (Gomendio et al., 1998). Preston and Stockley (2006) showed that males adjust their copulatory behaviour to female oestrus stage, suggesting that males can assess the timing of ovulation. If plugs represent a significant physical barrier to rival males, selection arising from post-copulatory competition is predicted to influence the male ability both to deposit efficient plugs in a defensive mating role and to remove plugs in an offensive mating role, possibly involving trade-offs between plug deposition and plug removal skills (Fromhage, 2012).

Sutter *et al.* (Chapter 4) recently showed that repeated ejaculation is accompanied by a decrease in plug size, and used the variation in plug size arising from the variation in time since a male's last ejaculation to investigate the effects on rival male behaviour and paternity share. Larger plugs tended to delay ejaculation by rival males and were associated with a larger first male advantage for paternity share. However, a small sample size due to pregnancy failure and nonindependence between plug size and sperm numbers limited the study's conclusions (Chapter 4). Here, we used a direct experimental approach to assess the role of the copulatory plug in sperm competition in house mice. After a female's first mating, we either removed (often only partially) the copulatory plug deposited by her first mate or left the plug intact. We then paired that female with another male and observed copulatory behaviour. Nine days into gestation, we killed females to obtain the estimates on paternity shares of the two competing males. We minimized the variation in sperm numbers and quality by using sexually rested full brothers of similar intrinsic sperm competitiveness. In the plug removal treatment, we removed the plugs deposited by both males in an attempt to control for the direct effects of plug removal on sperm numbers.

5.3 Material and Methods

5.3.1 Experimental animals

Experimental matings were performed using 86 male (aged 2–4 months) and 159 female (aged 2–5 months) laboratory-born F1 to F3 descendants from a free-living population of wild house

mice (*Mus musculus domesticus*) in Switzerland (see KÖNIG AND LINDHOLM 2012). Mice were bred and kept in standard laboratory conditions in a 14L:10D cycle (breeding colony: lights on at 05:30 CET; mating experiments: reversed cycle with lights on at 17:30 CET) at a temperature of 22–24 °C, with food (laboratory animal diet for mice and rats, no. 3430, Kliba) and water provided *ad libitum*. Experimental males were derived from 10 different monogamous breeding pairs where at least one parent (typically the male) had been caught in the free-living population; females were derived from 20 breeding pairs containing a mix of wild-caught and laboratory-born individuals. Offspring were weaned at 23 days after birth and kept in same-sex sibling groups in Macrolon Type III cages (23.5 × 39 × 15 cm). At latest at the onset of aggression between brothers, males were separated and kept individually in Macrolon Type II cages (18 × 24 × 14 cm). We moved mice into the room with the reversed light cycle to allow acclimatization for at least two weeks before being used in the experiment. Experimental procedures received ethics approval by the Veterinary Office Kanton Zurich, Switzerland (licence no. 110/2013) and were conducted in accordance with Swiss law.

5.3.2 Plug removal experiment

In controlled laboratory matings, we investigated the effect of experimental plug removal. Trials were started 2.5 h ± 0.5 (mean ± SD) after the beginning of the 10-h dark phase of the reversed light cycle (lights off at 7:30 CET) and were conducted under dim red light to allow video observation. We used virgin females in naturally cycling oestrus and followed a mating protocol described in CHAPTER 1. Briefly, a sexually receptive female (based on vaginal cytology; BYERS ET AL., 2012) was introduced into a male's cage after having removed some of the nesting material to facilitate video observation. Every 1–1.5 h, females were checked for the presence of a copulatory plug, indicating ejaculation by the male (RUGH, 1968). We checked for plugs by releasing the pair into a handling bin and briefly restraining the female to check her vagina under dim white light, before returning the pair into the cage. Thus, mice were out of their cage for approximately one minute during a check. Once a copulatory plug was detected, the plug was either experimentally removed by gently pressing the female against the edge of the handling bin and dislodging the plug with a blunt probe (FIRMAN & SIMMONS, 2008A), or females were sham treated. This control treatment included restraining the female and pressing her against the edge of the handling bin for a similar amount of time as in the plug removal treatment, without removing the copulatory plug. Plugs could often not be removed fully by gentle probing, resulting in partial plug removal in many of the trials. We visually estimated the extent of plug removal and weighed the removed piece of the plug to the nearest 0.1 mg (see SUPPLEMENT C). The female was then added to the cage of the first male's brother and checked every 30–60 min until a second copulatory plug was observed. Alternatively, if the pair had not mated before the end of the dark phase, the pair was left undisturbed throughout the light phase until the beginning of the next dark phase in order to allow ample time for mating. At the end of the trial, the plug was again either removed or the female was sham treated. Thus, females either had both or neither of their mates' plugs (partly) removed. The female was transferred into a clean cage containing nesting material and *ad libitum* food and water. Experimental trials that did not result in any mating were stopped at the end of the dark phase and females were re-tested on a later occasion. Males and females were weighed to the nearest 0.1 g before the start of mating trials. We used a paired design with individual males mating in the same order with and without experimental plug removal until we obtained at least one pregnant female from both of the treatments for a given brother pair (there were three

exceptions with only one pregnant female). Males were sexually rested for a minimum of three days between individual trials to allow sperm and seminal fluid replenishment (Chapter 4). To account for the potential order effects arising from using initially sexually naïve males, half of the brother pairs commenced in the plug removal treatment and half commenced in the control treatment.

This experiment was part of a series of experiments on reproductive behaviours in relation to the *t* haplotype, a selfish genetic element that shows segregation distortion in males and is frequently found in wild populations (Silver, 1993).). A tissue sample taken by earpunch at weaning was used for *t* haplotype genotyping and individual marking. DNA extraction was performed by salt–chloroform extraction (Müllenbach et al., 1989) and *t* genotype was diagnosed by PCR (Schimenti & Hammer, 1990; Lindholm et al., 2013). We have previously shown that males heterozygous for the *t* haplotype (+/*t*) are strongly disadvantaged in post-copulatory competition against wild-type (+/+) males (Chapter1). Here, we predominantly competed full brothers that were equal with respect to *t* genotype (+/+ vs. +/+ and +/*t* vs. +/*t*) against each other. In some trials however, brothers differed with respect to their *t* genotype. Because of the strong effect of the *t*haplotype on sperm competitiveness (Chapter1), these trials were not included for paternity analyses. Some of the females involved in these experiments also carried the *t*, but there is no evidence that female genotype at this locus influences the outcome of post-copulatory competition (Chapter1). We used full brothers from the same litter to minimize the genetic effects on sperm competitiveness other than the *t*. The experimenter was blind with respect to the mice's genotype during mating trials and their analyses.

5.3.3 Copulatory behaviour

Mating trials were conducted during the dark phase under red light spots. We used video recording with infrared night vision (Sony digital cameras DCR-SR40 and DCR-SR62) to quantify the copulatory behaviour and to confirm ejaculation by both males. Video observation also ensured that the observer was blind with respect to the experimental treatment when quantifying the behaviour. Copulatory behaviour of male mice is characterized by initial mounts, a variable number of mounts with intromission (during which the male inserts his penis and performs pelvic thrusts) and ejaculation including the deposition of the copulatory plug (McGill, 1962). One copulatory series includes all mounts and mounts with intromissions and ends with ejaculation. We collected the detailed behaviour of second-to-mate males but were only able to reliably distinguish between mounts without intromission and mounts with intromission in part of the trials because of visibility issues associated with the video observations. Thus, we recorded (i) the latency from introduction of the female until the first mount, (ii) the number of copulatory bouts (mounts and mounts with intromissions) until ejaculation, (iii) the duration of copulatory bouts, (iv) the latency to ejaculation (from the first mount) and (v) the duration of genital contact during ejaculation. The delay between the two competing males' ejaculations may influence the outcome of sperm competition. Hence, we noted (vi) the timing of ejaculation of both males. Similarly, because males sometimes perform two full copulatory series with the same female and the number of ejaculations influences paternity success (Chapters 1 & 4), we counted (vii) the number of ejaculations of both males. When first males perform a second copulatory series, they may be loosening their own previously deposited plugs. We recorded (viii) the number of post-ejaculatory copulatory bouts performed by the first male to investigate this possibility.

5.3.4 *Paternity assignment*

Paternity was assigned as described in CHAPTER 1. Briefly, we killed females 9 days *post coitum* using gradual CO_2 filling in their home cage and recovered all implanted embryos. We scored 12 microsatellites spread across 10 autosomes and performed paternity analysis at a confidence level of 95% with a single or no mismatch between offspring and assigned father in CERVUS (KALINOWSKI ET AL., 2007).

5.3.5 *Statistical analyses*

Sample sizes available for statistical analyses are summarized in Table 5.1. Data can be found on the Dryad data repository (doi:10.5061/dryad.676q3). Using R version 3.1.3 (R CORE TEAM, 2015), we analysed data on the occurrence of remating, copulatory behaviour, pregnancy rates and paternity outcome with either linear (LMM) or generalized linear mixed models (GLMMs), depending on the response variable. Analyses were performed using the functions lmer and glmer in lme4 (BATES ET AL., 2014). In all models, male identity nested within male family was included as a random factor to account for our paired design and to avoid pseudoreplication. Female family was associated with negligible variance for both behavioural variables and paternity share. Thus, it was not included as a random factor in the analyses presented. We obtained *P*-values for fixed effects in LMMs using F-tests, with degrees of freedom based on the Kenward–Roger approximation implemented in the package pbkrtest (HALEKOH & HØJSGAARD, 2014). To avoid biasing effect sizes through the removal of nonsignificant terms (FORSTMEIER & SCHIELZETH, 2011), we extracted the effect sizes from full models and calculated approximate confidence intervals by multiplying Student's t-values for our sample sizes by standard errors of the predicted values (CRAWLEY, 2007). To improve the interpretability, continuous input variables were standardized to a mean of 0 and a standard deviation of 1 as recommended by Schielzeth (2010). Figures show untransformed raw data as well as mean model predictions and approximate 95% confidence intervals (back-transformed to the original scale where appropriate and centred for nonfocal predictor variables).

Table 5.1

	Plug removal experiment		Additional matings	Total
	Control	Removed	*Post mortem removal*	
Females paired with male (N males)	100 (64)		59 (22)	159 (86)
Females mated (N males)	42 (31)	42 (32)	43 (20)	127 (83)
Females remated (N males)	33 (28)	37 (32)	–	70 (60)
Pregnant females (N paternity/N embryos)	28 (213/224)	30 (220/232)	–	58 (433/456)

Overview of sample sizes for different hierarchical levels of the experimental plug removal experiment and the additional matings (see SUPPLEMENT C). The number of individual females is indicated, with the number of individual males or embryos in brackets.

Experimental plug removal – Of 100 females used for mating trials, 84 females mated after an average of 1.9 trials (range 1–5). After 42 of these first matings, the copulatory plug was fully or partially removed. Difficulties with plug removal resulted in a large variation in the size of the removed piece of the plug (SUPPLEMENT C; Figure S1). Thus, we performed our analyses in two steps. First, we tested for an effect of copulatory plugs on male copulatory behaviour and paternity outcome, comparing control trials to trials with experimental plug removal as categories. In an extension, we focused on trials with experimental plug removal, where we used the weight of the plug piece removed as a continuous proxy for the size of the remaining plug (SUPPLEMENT C; Figure S1).

Copulatory behaviour – We tested for an effect of plug removal (control vs. plug removal) on remating with a binomial GLMM and on the six individual components of copulatory behaviour of second males with LMMs. We log-transformed mount latency data and square-root-transformed average bout duration, ejaculation latency and *in copula* duration at ejaculation to satisfy the assumptions of normal residuals and homoscedasticity. We fitted full models including the following variables as fixed effects: treatment (control vs. plug removal), the second male's body weight and female body weight and the number of post-ejaculatory copulatory bouts performed by the first male. Our full models included 65 mating trials for which we had complete information on all these variables.

To analyse the copulatory behaviour in a subset of the trials where we had more detailed information on mounts and mounts with intromissions ($N = 49$), we analysed the occurrence of intromission along the sequence (i.e. chronological order) of copulatory bouts. We ran a binomial GLMM on mounts with intromission vs. mounts (coded as 1 and 0, respectively). Experimental treatment, the sequence order of copulatory bouts (i.e. a value of 1 for the first bout, 2 for the second bout, etc.), their interaction as well as male and female body weight and the number of post-ejaculatory copulatory bouts performed by the first male were included as fixed effects. To account for repeated measurements (2096 copulatory bouts from 49 females mated to 26 different males from 9 families), we included random intercepts for female identity nested within male identity nested within male family. Random slopes for individual females were included to avoid overconfidence in the interaction term (SCHIELZETH & FORSTMEIER, 2009).

Pregnancy rates – Some of the mated females did not become pregnant. We analysed the potential effect of plug removal, remating and copulatory behaviour on pregnancy rates using GLMMs. The number of implanted embryos was analysed with LMMs.

Paternity share – Of 58 successful trials where the female mated with both males and became pregnant, 15 involved competition between $+/t$ and $+/+$ males, and previous research showed that $+/t$ males are strongly disadvantaged in sperm competition (CHAPTER 1). For the final paternity analyses, we thus reduced our data set to include only sperm competition trials between brothers of the same genotype (i.e. with similar intrinsic sperm competitiveness), because a paternity skew due to the *t* haplotype would have biased effect size estimates for plug removal. We analysed the paternity share of the first male (P_1) with binomial GLMMs. The number of embryos sired by the first male was included as the dependent variable and the number of offspring genotyped as the binomial denominator. To investigate how plug removal affects the paternity share, we ran a GLMM on P_1, including experimental treatment as well as the genotype combination of the brothers and the difference between the number of ejaculations of the first and second male as fixed effects. Continuous input variables were standardized.

Plug removal may affect the outcome of sperm competition indirectly by influencing rival male behaviour as well as directly by the physical removal of part of the ejaculate. Thus, we ran a multiple regression analysis on paternity outcome to investigate the relative importance of different explanatory variables. Experimental treatment, the interval between the first male's and the second male's first ejaculation (i.e. the duration of exclusive representation of the first male's ejaculate in the female reproductive tract), the difference in the number of ejaculations performed by both males, the difference in body weight between the two males as well as the *t* genotype of both males were included as fixed effects, and male identity was included as a random effect. Dispersion parameters of the GLMMs were ≈1.

5.4 Results

5.4.1 Experimental plug removal

To test the function of the copulatory plug, we introduced the variation in the presence or size of a first male's copulatory plug by removing as much of the plug as we could after ejaculation or leaving the plug intact. In plug removal trials, we removed 29.6 ± 13.7 mg (mean ± SD) of plug material (see Figure S1). Fully and partly removed plugs weighed 40.6 ± 6.4 mg and 27.1 ± 13.7 mg, respectively.

5.4.2 Copulatory behaviour

We investigated the effect of experimental plug removal on different aspects of copulatory behaviour of second-to-mate males. Of the 84 females that mated with the first male, 70 mated to ejaculation with the second male. The probability of ejaculation by the second male was not influenced by plug removal (control: 33/42; plug removal: 37/42; GLMM: $z = 1.15$, $P = 0.250$). Initial analyses showed that the treatment order (i.e. mating experience) of brother pairs did not have an effect on mating behaviour. Likewise, copulatory behaviour of $+/t$ males was not different from that of $+/+$ males (data not shown). Order and genotype were thus dropped from the subsequent models. An overview of the analysed behaviours and their associations with plug removal treatment is given inTable 5.2 and the effects are illustrated in Figure 5.1. Effect sizes, approximate 95% confidence intervals and *P*-values were obtained from full models, for which details are provided in Table 5.3. After adjusting *P*-values for false discovery rates in order to reduce the probability of obtaining false-positive results (Benjamini & Hochberg, 1995), plug removal had significantly negative effects on the number of copulatory bouts (Figure 5.1b) and ejaculation latency (Figure 5.1d), and a positive effect on the average duration of copulatory bouts (Figure 5.1c). Mount latency (Figure 5.1a) and in copula duration at ejaculation (Figure 5.1e) were not significantly different between control and plug removal trials. The timing of ejaculation relative to the change-over from a female's first to her second mate (‘male change-over’) did not differ between first males of the control vs. plug removal treatment ($F_{1,44} = 0.08$, $P = 0.773$; Figure 5.1g). For second-to-mate males, ejaculation timing was earlier in the plug removal treatment ($F_{1,44} = 5.78$, $P = 0.021$; Figure 5.1f).

We show a more detailed account of the effect of plug removal on copulatory behaviour in Figure 5.2. Plug removal was associated with longer copulatory bouts at the onset of mating trials, thus increasing the average bout duration relative to control trials. We hypothesized that this

early increase in copulatory bout duration was due to the full or partial removal of the physical barrier that the plug represents, and thus that there was a higher incidence of intromissions during these bouts. If so, these intromissions could have then facilitated more copulatory stimulation (and the removal of remains of previous plugs), potentially explaining the decrease in ejaculation latency in the plug removal group. To test this interpretation, we used more detailed information from a subset of the trials ($N = 49$), for which we had been able to distinguish between mounts and mounts with intromissions. The proportion of mounts with intromission increased along the sequence order of copulatory bouts (GLMM: 2096 observations, 49 females, 26 males, $z = 5.38$, $P < 0.001$; i.e. intromission was more likely during later copulatory bouts) and was lower for the control treatment (i.e. the proportion of intromissions was lower; $z = 4.00$, $P < 0.001$; Figure 5.2). The interaction between treatment and sequence order, male and female body weight and the number of post-ejaculatory copulatory bouts performed by the first male did not have significant effects (all $P > 0.246$). Thus, the increase in the proportion of mounts with intromission over time (sequence order of copulatory bouts) was similar for control and plug removal trials (top section in Figure 5.2).

5.4.3 Pregnancy rates

Of 84 females that received at least one ejaculation, 23 did not become pregnant. Experimental plug removal affected neither fertility nor fecundity. Pregnancy rates were not significantly different between the control and plug removal groups ($31/42 = 74\%$ vs. $30/42 = 71\%$; GLMM: $z = 0.25$, $P = 0.807$), and there was no difference in the number of implanted embryos per female in the two treatments (control: 8.0 ± 0.3; plug removal: 7.7 ± 0.4; LMM: $F_{1,32} = 0.32$, $P = 0.575$).

Females that had received an ejaculation by the first and second male were significantly more likely to become pregnant than females that had received no ejaculation by the second male (pregnancy rate remating: $58/70 = 83\%$; no remating: $3/14 = 21\%$; GLMM: 84 trials, 32 brother pairs, $z = 3.97$, $P < 0.001$), but a GLMM additionally including the number of copulatory bouts performed by the second male suggested that copulatory stimulation was more important for pregnancy than ejaculation *per se* (copulatory bouts: $z = 3.04$, $P = 0.002$; ejaculation: $z = 1.59$, $P = 0.113$; variance inflation factor = 1.3). Additional analyses on the behaviour of first-to-mate males showed that pregnant females had also received more copulatory stimulation by their first mates than nonpregnant females (mean $\pm$ SD: 18 ± 14 vs. 11 ± 13; LMM (log-transformed): $F_{1,75} = 10.12$, $P = 0.002$). In contrast, the number of implanted embryos was not affected by remating ($F_{1,58} = 0.01$, $P = 0.934$) or by the number of the second male's copulatory bouts ($F_{1,52} = 0.29$, $P = 0.590$)

5.4.4 Paternity share

The paternity share of first males (P_1) was significantly influenced by plug removal as well as by the difference in the number of ejaculations of the competing males and their genotype combination (Table 5.4). Thus, full or partial removal of the first male's copulatory plug reduced his paternity share (GLMM: 42 trials, 24 brother pairs, $z = -2.24$, $P = 0.025$, b [95% CI] $= -0.96$ $[-1.83, -0.10]$; Table 5.4). The effect of plug removal on P_1 could have either been indirect by influencing rival male ejaculation timing, or direct by physically affecting ejaculate components important for fertilization (e.g. sperm numbers). To investigate this possibility, we performed multiple regression on P_1. The full model showed the positive effects of both ejaculation interval and ejaculation numbers on paternity share (Fig. 5.3 and Table 5.4; GLMM: 41 trials, 24 brother

pairs; interval: $z = 3.44$, $P < 0.001$, $b = 1.31$ [0.54, 2.07]; ejaculation numbers: $z = 2.10$, $P = 0.035$, $b = 0.79$ [0.45, 2.00]), although the two variables showed some collinearity (variance inflation factor = 1.5). There was an additional significant effect of the *t*haplotype, with lower P_1 values when two $+/t$ brothers competed (Table 5.4). When controlling for these effects, an additional effect of plug removal on paternity outcome was not statistically supported ($z = -1.92$, $P = 0.233$, $b = -0.62$ [−1.68, 0.43]; Table 5.4).

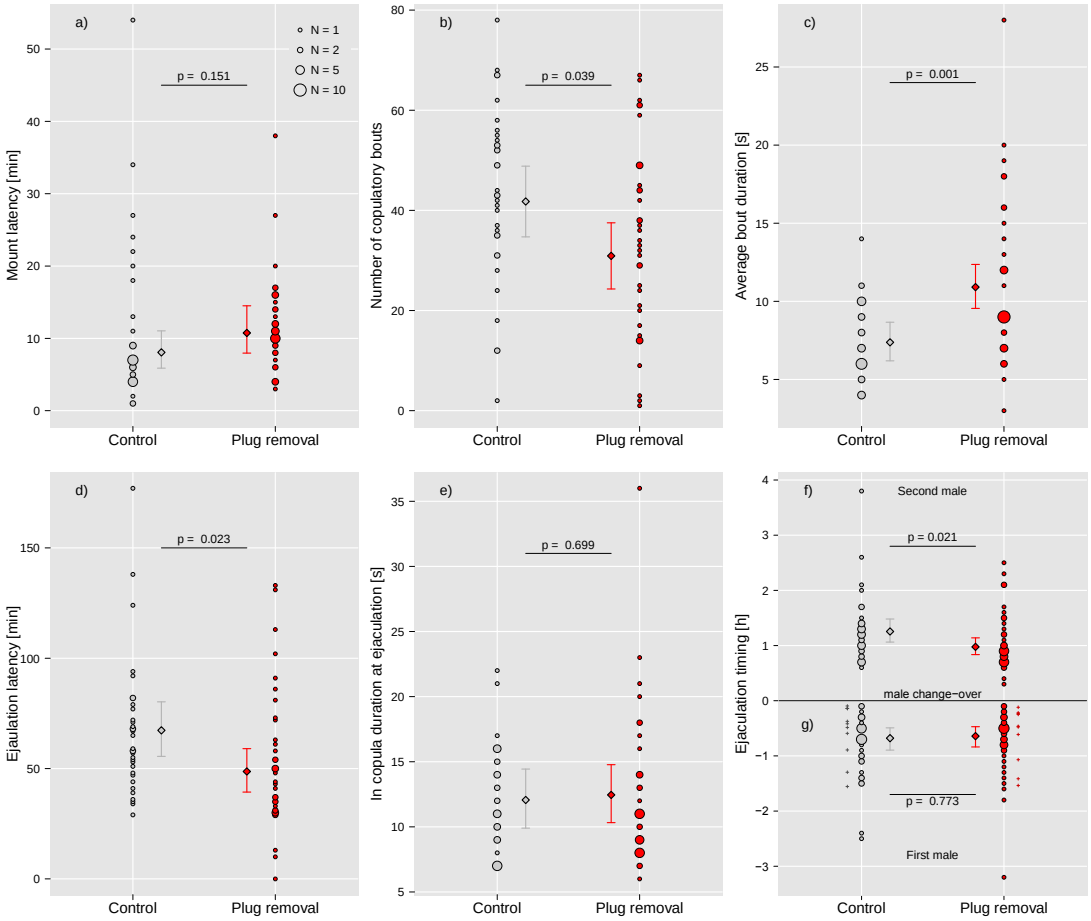

Figure 5.1

Copulatory behaviour of second-to-mate males for trials where the first male's plug had been left intact (control treatment; grey) versus trials where it had been fully or partly removed (plug removal treatment; red): (a) mount latency, (b) the number of copulatory bouts, (c) average duration of copulatory bouts, (d) ejaculation latency, (e) in copula duration at ejaculation, and (f) the timing of ejaculation relative to the change-over from a female's first to second mate. The timing of the first ejaculation of first males relative to male change-over (g) did not differ between the two treatment groups. Plus-symbols show second ejaculations by first males. The surface area of circles is proportional to the number of observations. Diamonds and error bars depict model predictions and approximate 95% confidence intervals obtained from full models (LMMs, back-transformed to the original scale when necessary; Table 5.2). P-values were corrected for false discovery rates to account for multiple testing (Benjamini & Hochberg, 1995).

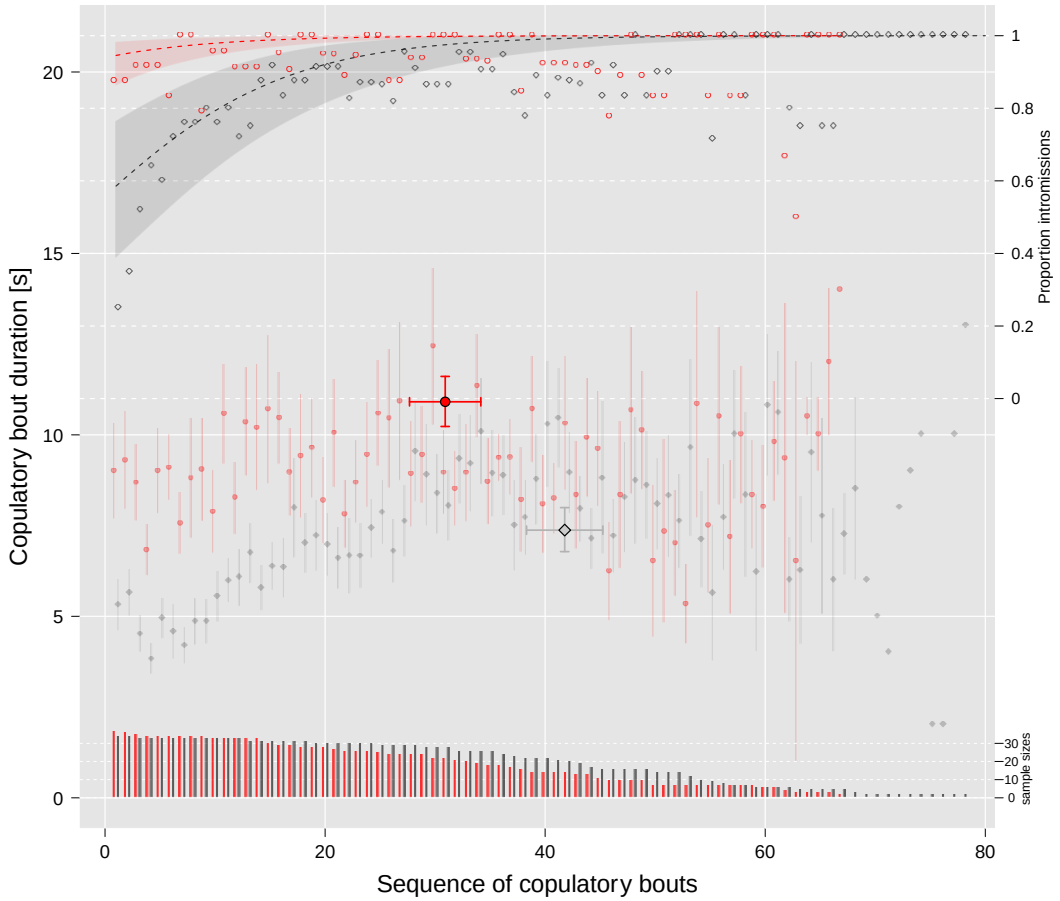

Figure 5.2

Copulatory behaviour in trials with copulatory plug removal (red circles) and control trials (grey diamonds). The mean duration +/- SE of copulatory bouts is shown along its chronological sequence (small filled symbols and error bars). Heights of the bars at the bottom of the figure indicate sample sizes. The large grey and red symbols and error bars represent full model predictions +/- SE for total number of copulatory bouts (X axis) and duration (Y axis). The number of copulatory bouts decreased as a function of plug removal, while mean bout duration increased with plug removal. Open symbols at the top of the figure show the proportion of bouts with intromission, analysed on a subset of the trials ($n = 49$). Dashed lines and shaded areas represent back-transformed model estimates and approximate 95% confidence intervals from a binomial GLMM on whether or not intromission occurred, with non-significant predictors removed (see main text). The proportion of copulatory bouts with intromissions increased over time, but was initially lower in control trials with intact plugs (top left), coinciding with shorter average bout duration (bottom left).

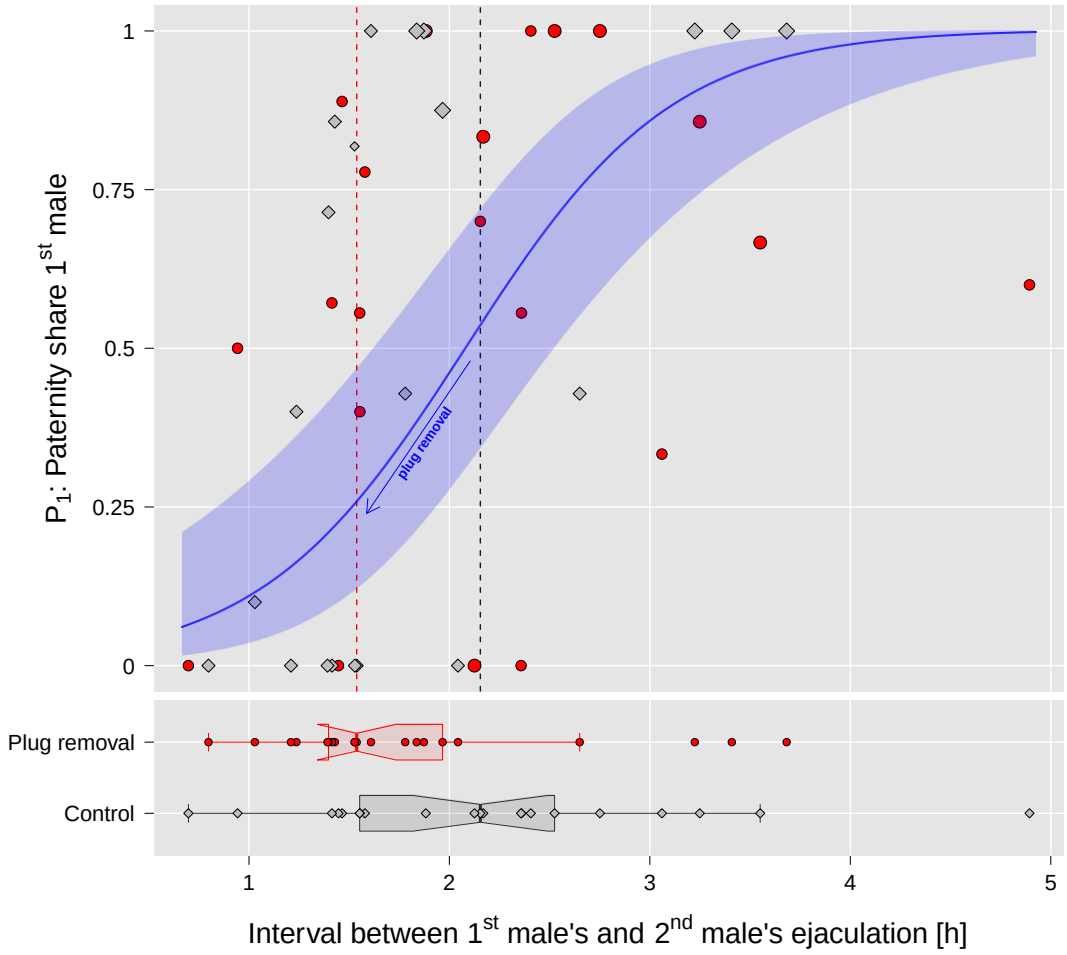

Figure 5.3

The effect of ejaculation delay on P1. Paternity share of the first male to mate (P1) is shown as a function of the interval between the first male's and the second male's first ejaculation (top panel). Trials with plug removal are represented by red circles and control trials by grey diamonds. The blue line and shaded area represent back-transformed mean and approximate 95% confidence interval estimates for the effect of ejaculation interval from a GLMM. The predicted effect is shown for when there is no difference in ejaculation numbers, and centred for plug removal, body weight difference and the competing males' genotypes. For illustrative purposes, large symbols represent two ejaculations by the first male. Dashed lines show medians of ejaculation intervals for control trials and trials with experimental plug removal (bottom panel). The blue arrow highlights the decrease in the ejaculation interval that is associated with plug removal, and the corresponding reduction in P_1.

Table 5.2

	mean ± SD (N = 69 trials)		Linear mixed models (N = 65 trials)			
Copulatory behaviour	Control	Plug removal	Tf	Effect size [95% CI]	p value	FDR-adjusted p
a) Mount latency [min]	10.8 ± 11.1	12.0 ± 6.5	Log	0.29 [-0.07, 0.64]	0.121	0.151
b) Number of copulatory bouts	43.2 ± 17.5	33.7 ± 18.7	–	**-10.9 [-19.9, -1.8]**	**0.024**	**0.039**
c) Average copulatory bout duration [s]	7.5 ± 2.5	11.0 ± 5.2	Sqrt	**0.59 [0.29, 0.88]**	**< 0.001**	**0.001**
d) Ejaculation latency [min]	67.3 ± 31.0	53.0 ± 31.4	Sqrt	**-1.23 [-2.11, -0.34]**	**0.009**	**0.023**
e) In copula duration at ejaculation [s]	12.0 ± 4.0	12.4 ± 5.9	Sqrt	0.06 [-0.22, 0.34]	0.699	0.699
f) 2nd male: Ejaculation timing [min] (changeover = 0)	78.1 ± 38.3	65.0 ± 32.1	Log	**-0.25 [-0.46, -0.05]**	**0.021**	– †
g) 1st male: Ejaculation timing [min] (changeover = 0)	-50.1 ± 34.6	-47.5 ± 36.3	Sqrt	0.18 [-1.01, 1.37]	0.773	– ‡

† FDR adjustment can produce more significant results when adding tests that exhibit an effect (Benjamini & Hochberg, 1995). Thus, excluding the test on ejaculation timing of the second male (=mount latency + ejaculation latency) was more conservative than including it.

‡ not included in the FDR adjustment because the concern here was a type II error rather than a type I error. Including this test in the FDR adjustment would have resulted in penalizing the positive results through a negative desired result (namely that the treatment groups did not differ prior to the manipulation).

Recorded copulatory behavioural traits and the influence of plug removal. Letters refer to the individual panels of Fig. 1. Mean and SD are given for the control group and plug removal group. Effect sizes and approximate 95% confidence intervals were obtained from full LMMs on untransformed or transformed (Tf) behavioural variables (see main text). We adjusted *P*-values for multiple testing using a correction for false discovery rates (Benjamini & Hochberg, 1995). Confidence intervals not overlapping zero and *P*-values smaller than 0.05 are highlighted in boldface. Plug removal affected the number of copulatory bouts, the average duration of the copulatory bouts, ejaculation latency and ejaculation timing (mount latency + ejaculation latency). The two treatment groups did not differ with respect to the ejaculation timing of the first-to-mate male.

Table 5.3

Model	Response variable Random effects	Fixed effects	Mean (SD)	Std?	Estimate [approx. 95% c.i.]	F value	p
LMM	Log(Mount latency [min])	Intercept			2.09 [1.78, 2.40]	–	–
	Male family/Male ID	Treatment (plug removal)	–	n	0.29 [-0.07, 0.64]	2.50	0.121
		Male body weight [g]	26.7 (2.3)	y	-0.12 [-0.31, 0.08]	1.28	0.265
		Female body weight [g]	20.8 (1.7)	y	0.03 [-0.15, 0.22]	0.10	0.756
		Post-ejaculatory bouts	2.5 (9.5)	n	-0.14 [-0.33, 0.05]	2.05	0.158
LMM	Number of copulatory bouts	Intercept			41.8 [34.9, 48.7]	–	–
	Male family/Male ID	Treatment (plug removal)	–	n	**-10.9 [-19.9, -1.8]**	5.48	**0.024**
		Male body weight [g]	26.7 (2.3)	y	2.3 [-2.2, 6.9]	0.88	0.355
		Female body weight [g]	20.8 (1.7)	y	0.9 [-3.7, 5.5]	0.13	0.715
		Post-ejaculatory bouts	2.5 (9.5)	n	0.20 [-0.28, 0.67]	0.59	0.445
LMM	Sqrt(Average bout duration [s])	Intercept			2.7 [2.5, 2.9]	–	–
	Male family/Male ID	Treatment (plug removal)	–	n	**0.59 [0.29, 0.88]**	15.43	**< 0.001**
		Male body weight [g]	26.7 (2.3)	y	**-0.17 [-0.32, -0.02]**	4.56	**0.040**
		Female body weight [g]	20.8 (1.7)	y	-0.08 [-0.23, 0.07]	0.93	0.340
		Post-ejaculatory bouts	2.5 (9.5)	n	-0.0003 [-0.02, 0.01]	0.002	0.969
LMM	Sqrt(Ejaculation latency [min])	Intercept			8.20 [7.47, 8.94]	–	–
	Male family/Male ID	Treatment (plug removal)	–	n	**-1.23 [-2.11, -0.34]**	7.43	**0.009**
		Male body weight [g]	26.7 (2.3)	y	**0.61 [0.10, 1.12]**	5.00	**0.031**
		Female body weight [g]	20.8 (1.7)	y	-0.42 [-0.91, 0.07]	2.52	0.118
		Post-ejaculatory bouts	2.5 (9.5)	n	-0.05 [-0.10, 0.001]	3.36	0.072
LMM	Log(In copula at ejaculation [s])	Intercept			3.47 [3.15, 3.79]	–	–
	Male family/Male ID	Treatment (plug removal)	–	n	0.06 [-0.22, 0.34]	0.15	0.699
		Male body weight [g]	26.7 (2.3)	y	0.04 [-0.14, 0.22]	0.15	0.698
		Female body weight [g]	20.8 (1.7)	y	-0.14 [-0.31, 0.02]	2.81	0.100
		Post-ejaculatory bouts	2.5 (9.5)	n	-0.01 [-0.03, 0.01]	1.65	0.205

Model summaries for full models on copulatory behavior. Response variables were transformed to satisfy model assumptions (sqrt = square root, log = natural logarithm). Mean and standard errors for fixed effects are given where appropriate. Weight variables were standardized (Std? = y) to improve interpretability. Degrees of freedom for F values were based on the Kenward-Roger approximation (Halekoh & Højsgaard, 2014). Confidence intervals not overlapping zero and p-values smaller than 0.05 are highlighted in boldface. LMM = linear mixed model.

Table 5.4

Response variable Random effects	Fixed effects	Mean (SD)	Std?	Estimate [approx. 95% c.i.]	Z value	P
P_1: Paternity share 1st male	Intercept			0.84 [-0.20, 1.88]	1.64	0.101
Male family/Male ID	Plug removal	–	n	**-0.96 [-1.83, -0.10]**	-2.24	**0.025**
	Ejaculation number difference	–	n	**1.64 [0.92, 2.36]**	4.59	**< 0.001**
	Male genotype combination	–	n	**-1.90 [-2.96, -0.84]**	-3.62	**< 0.001**
P_1: Paternity share 1st male	Intercept			0.65 [-0.27, 1.56]	1.43	0.154
Male family/Male ID	Plug removal	–	n	-0.62 [-1.68, 0.43]	-1.92	0.233
	Ejaculation interval [h]	2.0 (0.9)	y	**1.31 [0.54, 2.07]**	3.44	**< 0.001**
	Ejaculation number difference	–	n	**0.79 [0.05, 1.54]**	2.10	**0.035**
	Body weight difference [g]	-1.3 (2.3)	y	0.06 [-0.7, 0.84]	0.17	0.867
	Male genotype combination	–	n	**-2.05 [-3.00, -1.13]**	-4.38	**< 0.001**

Model summaries for models on sperm competition outcome. Mean and standard errors for fixed effects are given where appropriate. Weight variables were standardized (Std? = y) to improve interpretability. Confidence intervals not overlapping zero and p-values smaller than 0.05 are highlighted in boldface. GLMM = generalised linear mixed model.

5.5 Discussion

Copulatory plugs are produced by males in many different animal taxa and are commonly interpreted as an adaptation to sperm competition. However, direct empirical demonstrations of benefits of plugs in a sperm competition context remain scarce. Using experimental copulatory plug removal, here we show that copulatory plugs affect the rival males' copulatory behaviour and the outcome of sperm competition. The observed effects on the number and average duration of copulatory bouts and ejaculation latency indicate that copulatory plugs represent a physical barrier to rival males and that intact plugs are effective in delaying ejaculation by competitors. Multiple regression analysis on the outcome of sperm competition suggests that males benefit from plug deposition through delaying rival ejaculation: first males whose rival ejaculated later obtained a larger paternity share than males whose rival's ejaculation was less delayed.

5.5.1 The copulatory plug affects copulatory behaviour

To investigate the potential of copulatory plugs as mechanical barriers to female remating, we compared the experimental trials where we removed plugs (or parts thereof, due to difficulties with plug removal) after a female's first mating to control trials where plugs were left intact. Female remating was not affected by the plug removal. Overall, female remating rate was high (83%), similar to previous laboratory studies on wild-derived house mice (Rolland et al., 2003; Chapters 1 & 4; but see Ramm & Stockley, 2014). When we investigated the effects on copulatory behaviour in more detail, we found significant associations between certain aspects of copulatory behaviour and plug removal. Thus, the number and the length of copulatory bouts as well as ejaculation latency were altered when the first male's plug was fully or partly removed. Males facing the obstacle of intact plugs performed more but on average shorter copulatory bouts and ejaculated later (Figure 5.1). Plugs appeared to affect mainly early mating interactions, with second males in the control treatment performing initially shorter copulatory bouts. This coincided with a lower proportion of mounts with intromissions (left part of Figure 5.2), probably indicating that intact plugs represented a physical obstacle to intromission. It is likely that only mounts with intromissions are effective at removing copulatory plugs. When males faced intact plugs, they sometimes appeared unable to insert their penis, thus requiring more copulatory bouts and a longer time period before ejaculation. Ramm & Stockley (2014) found that males performed more intromissions and had a longer ejaculation latency when paired with mated (i.e. plugged) females than when paired with unmated females. Although there are potential explanations for this besides copulatory plugs (Ramm & Stockley, 2014), these effects are in agreement with what we found here. Collectively, available evidence suggests that although copulatory plugs do not prevent female remating, plugs represent physical obstacles that rival males have to remove before they can effectively deposit their own ejaculate and that plugs thus delay ejaculation by rival males.

The limitations of our experimental approach call for some caution when interpreting the observed effects. There may have been unintended differences between our control and treatment groups in addition to the difference in the presence or size of the plug. We were able to rule out the differences in the timing of the first male's ejaculation between the two experimental groups, but other aspects may have differed. Although an ideal sham treatment would have replicated all aspects of the plug removal treatment other than plug size or presence, copulatory

plugs cannot be removed and re-inserted. Thus, the physical plug removal might have caused more stress in females than the simple restraint in the control treatment. Indeed, mount latency was slightly longer in plug removal trials than in control trials, possibly indicating that it took longer for females to resume the normal behaviour. However, this difference was not statistically significant ($P = 0.121$). Moreover, although not statistically significant, a model based on the continuous variation of the removed part of the plug accurately predicted the observed ejaculation timing in control trials (see Figure S2). This suggests that the plug removal procedure *per se* did not alter the copulatory behaviour, at least with respect to the timing of ejaculation.

An unintended consequence of our difficulties with removing the entire plug was that the mean difference in plug size between the two experimental groups was less than expected. Plug removal trials may thus often have mimicked situations where smaller plugs are deposited (e.g. when males are not fully sexually rested; CHAPTER 4). Thus, a contrast between the presence and complete absence of a copulatory plug might result in more pronounced differences in the copulatory behaviour of rival males than those observed here.

5.5.2 Plug removal reduces P_1

Our paternity data showed that experimental plug removal affected the outcome of sperm competition. When copulatory plugs were experimentally removed, the paternity share of the first-to-mate male decreased. CHAPTER 4 showed that a first male's sexual rest (time since last ejaculation) affected the sperm competition outcome, but the experimental design did not separate plug size from ejaculate size. Here, we used sexually rested males and fully or partly removed their copulatory plugs, thus introducing the variation in the presence or size of the plug while minimizing the variation in sperm numbers. For our analyses on paternity outcome, we included only sperm competition trials between full brothers from the same litter. Moreover, we removed the plugs after each mating of a female, thus controlling for the potential direct effects of plug removal on nonplug ejaculate components such as sperm numbers. Indeed, additional analyses on the continuous variation in plug removal for first and second males showed no evidence for a negative effect of the extent of plug removal on sperm numbers (SUPPLEMENT C).

Using males with similar intrinsic sperm competitive abilities and observing copulatory behaviour in detail enabled us to focus on the effect of ejaculation timing on competitive fertilization success. Timing effects on paternity share have been demonstrated in hamsters, ground squirrels and rats (HUCK ET AL., 1989; SCHWAGMEYER & FOLTZ, 1990; CORIA-AVILA ET AL., 2004) with a longer delay of the second male's ejaculation leading to a greater paternity share for first males. Here, we confirm that the interval between the first male's and the second male's ejaculation is an important determinant of competitive fertilization success in house mice. Vaginal stimulation immediately after plug deposition has been shown to strongly reduce an ejaculate's fertilization potential in mice (BLOCH, 1972), hamsters (HUCK ET AL., 1989), and rats (ADLER & ZOLOTH, 1970; CORIA-AVILA ET AL., 2004). In our experiment, neither plug removal nor copulation with a second male immediately followed the first male's ejaculation. If females are exposed to males in immediate succession, copulatory plugs may prevent or reduce the likelihood of immediate vaginal stimulation after plug deposition, thus protecting a male's ejaculatory investment from rival males.

Besides timing, the number of ejaculations influences paternity in mice (CHAPTERS 1 & 4) and more generally in rodents (STOCKLEY & PRESTON, 2004). Our analyses showed that when accounting for

the ejaculation interval, the number of ejaculations showed an additional positive effect on P_1 (Table 5.4). However, as a consequence of our experimental design, there was some collinearity between ejaculation interval and the number of ejaculations performed by the first male. Females were left with their first mate for longer when he ejaculated twice and his first ejaculation was not detected during a cage check (as he had dislodged his own plug). This collinearity somewhat limited our ability to disentangle the relative importance of ejaculation number and ejaculation interval.

5.5.3 Evolutionary implications

We identified fitness-relevant effects of copulatory plugs on house mouse sperm competition that may help explain the evolution and persistence of copulatory plugs. Intact plugs probably benefited first males by delaying rival male ejaculation, which was associated with a larger paternity share. Given that the response in copulatory behaviour to plugs was mainly seen during the first third of copulatory bouts (Figure 5.2), it is somewhat surprising that second males did not ejaculate sooner, considering the negative effect of ejaculatory delay on their paternity share (Figure 5.3). However, copulatory stimulation may also increase sperm numbers within an ejaculate (Toner & Adler, 1986), thus affecting its competitiveness. Here, we found that females with more copulatory interactions with second males were more likely to become pregnant. Moreover, females that did not become pregnant had also received less copulatory stimulation by their first mates. Importantly, pregnancy failure was not different between our two treatment groups, thus not biasing the observed reduction in P_1 associated with the plug removal. The observational nature of our data prevents us from identifying the causation of pregnancy failure. Nonetheless, if the substantial copulatory stimulation is required for pregnancy in mice (deCatanzaro, 1991; cf. Dewsbury, 1979), pregnancy initiation might represent a potential additional incentive for males to maintain a high number of copulations and potentially for females to mate with more than one male. The ejaculation latency observed might reflect a male trade-off between increasing copulatory stimulation and reducing ejaculatory delay. An alternative explanation for the negative association between copulatory stimulation and pregnancy failure may be male coercion. Females may have attempted to discriminate against certain second mates by avoiding copulation. The laboratory setting of our mating trials prevented females from escaping, thus potentially allowing males to enforce copulation and ejaculation. Females may then have resorted to discriminating against these males by not initiating or aborting pregnancy (Eberhard, 2009).

In our laboratory set-up, second males were separated from females typically after a single ejaculation. If a longer ejaculation latency induced by a larger plug increases the chance of aggressive takeover by other males or reduces the length of the remaining period of female sexual receptivity available to perform a second ejaculation, the importance of the copulatory plug for competitive paternity success may be even more pronounced in a natural setting. Preston and Stockley (2006) found that males were less likely to ejaculate twice if they had provided more copulatory stimulation to females during their first ejaculatory series, providing support for a reduced likelihood of ejaculating twice when ejaculation latency is long. In our set-up, males were also fully sexually rested and thus able to produce large plugs. With repeated ejaculation, males become limited in sperm and in seminal fluids required for the copulatory plug (Chapter 4). Similar to sperm limitation, seminal fluid limitation may lead to a reduction in paternity skew, when mating with a larger number of females leads to a decrease in post-copulatory competitiveness in each mating event (Preston et al., 2001).

Many accounts of copulatory plugs have regarded them as adaptations to sperm competition, but the focus is often put on their potential to prevent remating (Fromhage, 2012). Given the differences in the evolutionary interests of the different actors involved in determining plug deposition and removal efficacy, copulatory plugs are unlikely to end up in a situation where they are completely ineffective or effective. Instead, the interplay between rival males and females will commonly lead to sexual conflict (Stockley, 1997; Friesen et al., 2016), intermediate plug effectiveness and evolutionarily dynamic changes. Here, we show that copulatory plugs that are ineffective at preventing female remating can still benefit their producers in a sperm competition context through subtle changes in rival male behaviour (Parker, 1970). Our results contribute to our understanding of the complex dynamics of copulatory plugs in house mice (Mangels et al., 2015), and highlight the importance for investigating fitness consequences of male traits at different stages of reproductive competition.

5.5.4 Concluding remarks

By experimentally manipulating copulatory plugs, we show that intact plugs represent a barrier to the subsequent rival males, delaying their ejaculation. A delay in rival male ejaculation was associated with a larger paternity share for plug producers, conveying a potential fitness benefit of depositing copulatory plugs. If this competitive benefit increases with plug size, this may result in directional selection for larger plugs and for larger plug-producing accessory glands in the presence of sperm competition.

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General discussion

The findings of this thesis can be considered under three aspects of sexual selection: the impact of meiotic drive on sperm competitiveness, fitness benefits of polyandry, and consequences for reproductive strategies. It is important to note that the very nature of these aspects is one of dynamic interactions between individuals and between the genes controlling the behaviours. Inevitably, many of the conclusions are extrapolations of results of a few experiments, and some conclusions are rather suggestive. Hopefully, these will stimulate future research that will confirm and/or expand on the findings presented here.

Impact of meiotic drive on sperm competitiveness

There is strong evidence that $+/t$ males are strongly disadvantaged in sperm competition and sire a drastically reduced number of offspring when competing against $+/+$ males (Chapter 1). The presence of the *t* haplotype also shows an unexpected interaction with male mating order. When two $+/t$ males compete against each other, the species-typical first male benefit is inversed to a second male benefit (Chapter 2). The sperm precedence pattern suggests that $+/t$ paternity success is related to the timing of mating relative to ovulation, and to premature hyperactivation of $+/t$ ejaculates (Chapter 2). Similar to the *t* haplotype, other meiotic drivers have revealed peculiar sperm precedence patterns (Atlan et al., 2004; Price et al., 2008a) that may further our understanding of sperm competition mechanisms and their dependence on mating order and timing (Pizzari et al., 2008; Smith, 2012; Bakker et al., 2014; Giraldo-Perez et al., 2016).

In contrast to paternity share, the strength of drive was unaffected by mating order (Chapter 3). Although the molecular mechanism of drive is well understood, the details of how drive acts in vivo are not fully understood. Sperm transport and fertilisation are complex processes, especially in internally fertilising species such as mammals (Suarez & Pacey, 2006; Suarez, 2008a). Besides evolutionary geneticists, the *t* haplotype has attracted much research on its impact on male fertility (Olds-Clarke, 1997), and it may well continue to be a useful tool for understanding mammalian fertility.

There is some potential for an additional connection between male meiotic drive and sperm competition. On the one hand, inter-ejaculate sperm competition limits the spread of drivers that manipulate intra-ejaculate competition. Sperm competition will thus halt the spread of male drivers. On the other hand, increased sperm competition will also favour increased sperm production (Hosken & Ward, 2001; Firman & Simmons, 2008b; Firman et al., 2015a). If increased cell proliferation is accompanied by a decrease in cellular policing (Lewis et al., 2008), this could in turn favour the rise of new male meiotic drivers.

The benefits of polyandry

Females that mate with multiple males can avoid costly fertilisation by +/*t* males and benefit from increased offspring viability. As a consequence of genetic incompatibility, benefits of increased offspring viability are limited to +/*t* females (Chapter 1). If the benefits of polyandry are purely non-additive, then the benefits are *t*-frequency-dependent and would not be able to favour the evolution of polyandry (see Champion de Crespigny et al., 2008). As discussed in Chapters 1 & 3, additional additive fitness effects of the *t* haplotype seem plausible, especially in natural populations. Currently, it remains unclear whether there is cryptic female discrimination against +/*t* males in this mouse population (Chapters 1 & 3; Lindholm et al., 2013). Importantly, cryptic female choice need not result in differences between +/*t* and +/+ females. It is possible that selection has favoured physiological adaptations in females that accentuate the sperm competition disadvantage of +/*t* males irrespective of female genotype. However, it might be interesting to ask how the female genetic background affects the outcome of sperm competition between +/*t* and +/+ males, i.e., whether females from populations or strains that have had a long coevolutionary history with the *t* haplotype have stronger negative effects on +/*t* sperm competitiveness.

Although there was no evidence for differences between +/*t* and +/+ females with respect to cryptic female choice, the finding that +/*t* females were less likely to mate (Chapter 3) is in line with previous reports of differences between +/*t* and +/+ females (Lenington & Franks, 1985; Franks & Lenington, 1986; Drickamer & Lenington, 1995; Manser et al., 2011; Auclair et al., 2013). These results suggest that females may follow different life history strategies, but the adaptive significance of this remains elusive. On a related note, this thesis has facilitated ongoing and future work on the genomics of the *t* haplotype. Many questions related to differences between +/*t* and +/+ mice and to the evolutionary history of the *t* haplotype will greatly benefit from next generation sequencing techniques. Sequencing the *t* haplotype is associated with many technical limitations, due to its size, the presence of major inversions, and early homozygote lethality. The experiments described here have yielded a number of *t/t* embryos that have proven very valuable for sequencing endeavours. It appears that the *t* haplotype encompasses many more genes than previously thought (Lindholm, unpublished data), with exciting new possibilities to identify potential candidate genes that will advance our understanding of the drive mechanism, and of physiological and behavioural differences between +/*t* and +/+ females that may reflect life history traits.

Male meiotic drive offers a heritable basis for sperm competitiveness. Additive genetic variation is a prerequisite for some of the hypotheses for the evolution of polyandry (e.g., the 'sexy sperm' hypothesis (Keller & Reeve, 1995); but see Bocedi & Reid, 2015), but heritability of polyandry is not commonly detected in empirical studies (Travers et al., 2016). One of the issues is that polyandry can deplete variation in sperm competitiveness if multiple mating rates are high. The ubiquity of intra-genomic conflict may constantly generate new drive systems in males and thus may offer a source of genetic variation in sperm competitiveness. If sexy sperm benefits are important, then male meiotic drive could favour the evolution of polyandry, irrespective of other costs of the driver such as homozygote lethality or sex ratio distortion.

Female preference and how to measure it are much-debated topics in sexual selection research (see Chapter 3). The results of this thesis demonstrate that inference of female preference or mating behaviour based on paternity patterns may not be possible or at least has to be made

with great caution (Dean et al., 2006). In the experiments presented here, the incidence of multiple paternity was overall low (37%), despite sampling embryos early during gestation and thus obtaining estimates from a relatively large number of offspring. In competition between +/*t* and +/+ males, only 28% (17/60) of the females that had mated with both males showed mixed paternity. This is strikingly similar to a recent lab study where females had simultaneous access to a +/*t* and +/+ male, and where multiple paternity was detected in 29% (19/34) of the litters (Manser et al., 2015). Arguably, in these two examples paternity was strongly skewed mainly due to the strong disadvantage of +/*t* males in sperm competition. However, even when the competing males were littermates of the same *t* genotype (Chapter 2) and performed the same number of ejaculations, multiple paternity was detected in only 57% (16/28) of the females. Differences in ejaculate size and intrinsic sperm competitiveness will further increase competitive skew under more natural conditions. These findings demonstrate how purely postcopulatory processes may explain lower reproductive success of +/*t* males in the wild, and how in general, multiple paternity may be a poor indicator of female mating behaviour. Experiments in semi-natural environments have yielded average multiple paternity rates between 20% and 70% (Carroll et al., 2004; Firman, 2011; Montero et al., 2013; Stockley et al., 2013), and wild populations show slightly lower but considerable rates of multiple paternity (around 20–45%; Dean et al., 2006; Firman & Simmons, 2008b; Lindholm et al., 2013; Thonhauser et al., 2014; Manser, 2015). It is likely that competitive skew is high in wild populations, too, and polyandry estimates based on multiple paternity could be strongly underestimating multiple mating rates (Dean et al., 2006). This limitation may be more severe than commonly appreciated, especially when the average number of offspring sampled per female is small. In the wild population that the mice used in this thesis were derived from (König & Lindholm, 2012), high-resolution movement data is being collected in combination with close monitoring of reproduction (König et al., 2015). This may offer an unprecedented possibility to improve our understanding of the link between male mating success and paternity success in a more natural context.

Consequences for reproductive strategies

The findings of this thesis reveal different potential strategies of how males can improve their reproductive success, but also highlight how trade-offs between alternative strategies, and conflict between males and between the sexes may constrain investing into a given strategy. A male can increase his competitive fertilisation success by ejaculating repeatedly with the same female (Chapters 1, 4 & 5). On the other hand, males are strongly limited in seminal fluids (Chapter 4) and thus in the number of females they can inseminate (Huber et al., 1980). Similarly, depositing a large copulatory plug at ejaculation can delay ejaculation by the rival male and increase competitive fertilisation success (Chapters 4 & 5), but it does not prevent a female from remating with a rival male. This is possibly because females can gain benefits from multiple mating and will counteract male manipulation (Stockley, 1997; Mangels et al., 2015). With regards to the *t* haplotype, this may prevent +/*t* males from maximising their reproductive success (Chapter 2). If *t* haplotype associated fitness costs are relevant enough to females, any male strategy should be counteracted by females (Alonzo, 2008). If +/*t* males were more likely to be aggressive and dominant as suggested by some studies (Franks & Lenington, 1986; Lenington et al., 1996), this could explain why females mate both with dominant and subordinate males (Rolland et al., 2003; Neff, 2008). One of the outstanding questions is whether female mating behaviour responds to selection pressures associated with the *t* haplotype, as was shown in experimental evolution for sex ratio drive in

Drosophila pseudoobscura (Price et al., 2008b). Alternatively, female house mice may be polyandrous for a number of other reasons (Jennions & Petrie, 2000) such as paternity confusion (Auclair et al., 2014) and protection from *t* haplotype associated fitness costs is a mere by-product of female multiple mating.

Similarly to conflict between the sexes, male-male competition could result in complicated evolutionary dynamics between plug deposition and plug removal skills (Fromhage, 2012) that could prevent males from reaching their optimum in either mating order. The effectiveness of any male strategy will thus depend on the strategy played by other males, and by how females actively influence the pay-offs of the different strategies (Alonzo, 2008).

Finally, intra-genomic conflict over controlling the behaviour of the gene collective will persist. To that end, it is curious that there appear to be no genetic suppressors present in wild house mouse populations. It is possible that spermatogenesis genes are coadapted gene complexes located in close proximity. In the example of the *t* haplotype, a number of genes crucial for sperm motility regulation are located within a rather small genomic region, so that the *t* haplotype was able to recruit a number of distorters through chromosomal inversions. If the genes regulating the sperm motility kinase that the *t* haplotype targets are all located within the same genomic region, genetic suppression might be too easy to counteract by the *t* haplotype by additional modifiers and inversions. In contrast, complex behavioural traits such as mating behaviour are probably highly polygenic and thus impossible to control by the *t* haplotype. This might make polygenic traits more suitable as a means to suppress the selfish action of a few alleles. Undoubtedly, more drive systems will be detected in the near future and will help shed light on common patterns of how meiotic drive arises and persists.

Concluding remarks

The results of the experiments outlined in this thesis have provided some compelling evidence for a strong effect of the *t* haplotype on sexual selection processes, and highlight the potential for a considerable impact of sexual selection on *t* haplotype frequencies in wild populations. More generally, this thesis reiterates that sexual selection, particularly postcopulatory selection, can influence evolutionary dynamics at multiple levels of selection. There is now mounting evidence that meiotic drive in males has strong negative consequences on sperm competitiveness, and that polyandry can greatly reduce the spread of meiotic drive. However, questions about how intra-genomic conflict can shape the mating system of populations are much more difficult to answer and require multi-generational approaches (e.g., Charlat et al., 2007; Price et al., 2008b, 2010). Many important questions about what limits and maintains meiotic drive in wild populations remain unanswered (Lindholm et al., 2016). Using the knowledge from controlled laboratory experiments should allow future research to gradually increase the complexity of questions addressed and decrease the amount of experimental control imposed.

SUPPLEMENT A – DETRIMENTAL EFFECTS OF AN AUTOSOMAL SELFISH GENETIC ELEMENT ON SPERM COMPETITIVENESS IN HOUSE MICE

SUPPLEMENTARY MATERIAL AND METHODS

Mating design

Whenever possible, we used *+/t* and +/+ full brothers from the same litter to minimise genetic effects on sperm competitiveness other than the *t* haplotype (40/45 male pairs). To account for order effects (1st male precedence in house mice; FIRMAN & SIMMONS, 2008A) and the *t* genotype of the female, we conducted up to four trials with each brother pair using a balanced mating order across trials. Female mice were virgins and males were virgins at the beginning of the experiment. We followed a mating protocol modified after (FIRMAN & SIMMONS, 2008A). Females were checked for estrous at the beginning of the dark phase, based on visual appearance of the vagina (CHAMPLIN ET AL., 1973) and on vaginal smears using plastic inoculation loops (modified after BYERS ET AL., 2012). Females in pro-oestrous or oestrous were paired with an unfamiliar non-sibling male in his cage after having removed some of the nesting material to facilitate video observation. Females were checked every 1–1.5 hours for the presence of a copulatory plug, indicating ejaculation by the male (RUGH, 1968). Once a copulatory plug was detected, the female was removed from the first male's cage, added to the second male's cage and checked every 30–60 minutes until either a second copulatory plug was observed or until the beginning of the next dark phase. After removal, the female was transferred into a clean cage containing nesting material and *ad libitum* food and water. We used the video recordings to confirm ejaculation by the second male and to count the number of ejaculations by both males. Ejaculation in house mice occurs after a variable number of mounts with penile intromissions and is characterised by a increase in pelvic thrust frequency, a final 'shudder' and a phase of immobility, during which the male usually tips over to his side (MCGILL, 1962). Males show a refractory period of around 30 minutes following ejaculation (ESTEP ET AL., 1975). Trials in which no plug by the first male was detected were stopped at the end of the dark phase and females were re-tested on a later occasion.

Paternity assignment

Most *t* haplotypes carry recessive lethal mutations leading to embryonic death and resorption of *t* homozygous offspring (SAFRONOVA, 2009). In the population we study, there is only a single lethal variant of the *t* haplotype, showing 90% drive in males and Mendelian inheritance in females (LINDHOLM ET AL., 2013). In several years of genotyping the free-living population and captive-bred mice descending from the same population, we have never found a *t* homozygous individual. Embryonic death of *t/t* embryos occurs early during gestation (A. Sutter, personal observation). Thus, we sacrificed females 9 days (+/- 1 day) after mating using gradual CO_2 filling in their home cage. Embryos were recovered under a dissection microscope and stored in 100% EtOH. For paternity assignment we scored 12 microsatellites spread across 10 autosomes (D3Mit278, D4Mit227, D5Mit122, D5Mit352, D6Mit139, D6Mit390, Chr8_3, D10Mit230, D11Mit90, D14Mit44, D16Mit139, and Chr19_17). Marker and PCR reaction details are described elsewhere (BULT ET AL., 2008; TESCHKE ET AL., 2008; LINDHOLM ET AL., 2013). Paternity analysis using the known mother and the two candidate fathers was performed using the software CERVUS (KALINOWSKI ET AL., 2007)

and a genotyping error rate of 0.01 (LINDHOLM ET AL., 2013). Paternity assignments were accepted at a confidence level of 95% with a single or no mismatch between offspring and assigned father.

Sample Sizes

Table S1

	+/*t* females		**+/+ females**		**total**
paired with male	51		89		140
mated	31		64		95
pregnant	24		54		78
remating unknown	1		5		6
first mate	+/*t* male	+/+ male	+/*t* male	+/+ male	
monandrous	3 (23)	2 (18)	5 (43)	5 (40)	15 (124)
polyandrous	11 (84)	8 (61)	21 (162)	17 (133)	57 (440)
# ejaculations known	7 (55)	5 (36)	15 (117)	14 (112)	41 (320)

Overview of sample sizes by female genotype and male mating order with the corresponding number of embryos with known paternity shown in brackets. Monandrous females did not remate and hence only received sperm from their first mate.

SUPPLEMENT B – MEIOTIC DRIVE CHANGES SPERM PRECEDENCE PATTERNS IN HOUSE MICE: POTENTIAL FOR MALE ALTERNATIVE MATING TACTICS?

SUPPLEMENTARY MATERIAL AND METHODS

Computer assisted sperm analysis (CASA)

We used full siblings from the same litter to account for potential environmental and genetic effects other than the *t* haplotype on sperm features. Sperm of brothers were measured on the same day to account for potential temporal variation in methodology and measurement instruments and the order of males within a brother pair was randomised. We used 24 sexually mature males (+/+ and +/*t* littermates) at 10–14 weeks of age. Males were different from the ones used in the sperm competition experiment and were either sexually naïve (14 males) or had been given up to eight mating opportunities and had mated between one and four times as part of a different experiment (10 males). Males with sexual experience were sexually rested for at least a week before their sperm were analysed. We sacrificed males using gradual CO_2 filling in their home cage, and dissected and weighed both caudae epididymides. We pooled both caudae and made multiple incisions with fine scissors in a 1mL drop of pre-warmed modified human tubal fluid (mHTF; Bühlmann Laboratories AG) immersed under mineral oil. Samples were incubated in 37 °C, and epididymal tissue was removed after 10 min. From each sample, 4–5 measurements were taken at different incubation times along a time series (at 15 min, 1 h, and at 2–6 h). We used computer assisted sperm analysis (CASA; MouseTraxx, Hamilton Thorne) to quantify sperm motility parameters. Approximately 7 µl of the sperm suspension was loaded into both chambers of a prewarmed improved Neubauer hemocytometer with a chamber depth of 100µm and scanned at 100x magnification. We performed four replicate scans from both chambers of the hemocytometer (one exception: only one chamber measured) for every sample at each time point, so that 327 ± 270 (mean $\pm$ SD) motile sperm were recorded for every sample per time point. The sperm suspension was diluted 1:1 with 50µl of pre-warmed mHTF if sperm concentration determined too high by the CASA system. We used the default mouse settings with minor adjustments (30 frames at 60 Hz; minimum contrast 50, minimum cell size 8 pixels) and recorded average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), and linearity (LIN). Individual files with track details were generated for every male at every time point.

Supplementary Results

Copulatory behaviour

Table S2

Behavioural variable	Mean ± SD		p_{full}	p_{geno}
	$+/t$	$+/+$	$n = 75$	$n = 83$
Time to first mount (mount latency) [min]	299 ± 97	286 ± 103	0.843	0.456
Number of copulatory bouts	13.3 ± 11.7	18.3 ± 15.4	0.085	0.254
Average duration of copulatory bouts [s]	18.4 ± 10.1	17.2 ± 7.0	0.811	0.479
Latency to ejaculation [min]	65.2 ± 38.1	70.2 ± 40.3	0.081	0.567
In copula duration at ejaculation [s]	15.8 ± 5.4	14.5 ± 4.3	0.842	0.355
Post-ejaculation interval [min]	26.1 ± 13.3	23.4 ± 11.2	–	0.576

Copulatory behaviour of first-to-mate males, their variability indices for $+/t$ and $+/+$ males, p-values (p_{full}) from likelihood ratio tests on the null hypothesis that behaviour was unaffected by any of the fixed effects (male and female *t* haplotype genotype and weight, oestrus stage and its interaction with male genotype), as well as p-values from univariate models on copulatory behaviour as a function of male genotype (p_{geno}). The p-value for post-ejaculation interval was obtained from a cox proportional hazard model that included right-censored data (see Chapter 2).

Principal component analysis of sperm features

Table S3

Sperm feature	*Mean*	*SD*	*PC1*	*PC2*
Average path velocity (VAP)	143.8	43.2	**0.737**	**0.523**
Straight-line velocity (VSL)	105.4	54.1	**0.953**	0.270
Curvilinear velocity (VCL)	262.6	60.4	0.331	**0.839**
Amplitude of lateral head displacement (ALH)	12.8	3.7	-0.346	**0.828**
Beat cross frequency (BCF)	23.7	12.9	-0.113	-0.378
Straightness (STR)	69.6	23.7	**0.910**	0.037
Linearity (LIN)	39.4	18.1	**0.972**	-0.066
Sums of squares of loadings	–	–	3.46	1.89
Proportion of variance explained	–	–	49.5%	26.9%

Recorded sperm features (subset of upper 50% sperm based on curvilinear velocity), their variability indices and results from a principal component analysis (PCA). The number of extracted components was determined using parallel analysis. Components were rotated using the varimax method and scores were calculated using regression. Variable loadings of more than 0.4 were considered interpretable and are highlighted in bold (Budaev, 2010).

Table S4

	VAP	VSL	VCL	ALH	BCF	STR	LIN
VAP	1	0.849	0.744	0.073	-0.127	0.523	0.650
VSL	0.849	1	0.551	-0.099	-0.154	0.866	0.901
VCL	0.744	0.551	1	0.460	-0.105	0.278	0.184
ALH	0.073	-0.099	0.460	1	-0.258	-0.165	-0.315
BCF	-0.127	-0.154	-0.105	-0.258	1	-0.203	-0.156
STR	0.523	0.866	0.278	-0.165	-0.203	1	0.909
LIN	0.650	0.901	0.184	-0.315	-0.156	0.909	1

Correlation matrix for the sperm features included in the principal components analysis. Sperm measurements were based on 12'614 sperm from 828 scans at 4–5 time points for each of 24 males.
VAP = Average path velocity; VSL = Straight-line velocity; VCL = Curvilinear velocity; ALH = Average lateral head displacement; BCF = Beat cross frequency; STR = Straightness; LIn = Linearity

Table S5

Genotype	Incubation [h]	VAP	VSL	VCL	ALH	BCF	STR	LIN	% Motile	Count
	0.25	166 ± 8	134 ± 8	293 ± 13	13 ± 0.4	33 ± 0.9	76 ± 2	44 ± 2	46 ± 5	65 ± 10
	1	177 ± 6	148 ± 7	292 ± 9	12 ± 0.3	33 ± 0.9	81 ± 2	51 ± 2	46 ± 5	53 ± 7
	2	154 ± 17	126 ± 19	261 ± 19	11 ± 0.3	33 ± 1.4	76 ± 6	46 ± 5	37 ± 12	53 ± 10
+/+	3	165 ± 6	136 ± 8	283 ± 11	12 ± 0.4	32 ± 1.4	81 ± 2	48 ± 2	49 ± 8	58 ± 16
	4	163 ± 8	137 ± 9	277 ± 6	12 ± 0.3	28 ± 1.3	82 ± 2	50 ± 3	49 ± 8	45 ± 9
	5	148 ± 6	120 ± 6	263 ± 12	12 ± 0.5	29 ± 0.5	77 ± 2	45 ± 1	36 ± 7	57 ± 11
	6	139 ± 7	107 ± 7	248 ± 9	12 ± 0.6	31 ± 1.8	74 ± 3	43 ± 2	53 ± 10	68 ± 9
	0.25	153 ± 7	117 ± 8	300 ± 8	14 ± 0.3	32 ± 0.6	72 ± 2	38 ± 2	53 ± 5	82 ± 9
	1	144 ± 7	109 ± 8	267 ± 9	13 ± 0.4	31 ± 0.5	71 ± 3	40 ± 2	61 ± 6	82 ± 10
	2	128 ± 5	96 ± 8	236 ± 4	12 ± 0.3	32 ± 0.7	71 ± 4	40 ± 3	61 ± 9	89 ± 20
+/*t*	3	144 ± 6	105 ± 11	255 ± 7	13 ± 0.5	31 ± 1.0	70 ± 5	41 ± 4	69 ± 6	72 ± 14
	4	131 ± 7	96 ± 8	242 ± 9	12 ± 0.3	30 ± 1.1	70 ± 4	39 ± 3	66 ± 9	95 ± 23
	5	134 ± 3	94 ± 7	241 ± 5	13 ± 0.2	30 ± 0.6	68 ± 4	39 ± 3	72 ± 7	98 ± 19
	6	120 ± 6	78 ± 7	219 ± 7	12 ± 0.5	29 ± 1.2	61 ± 3	34 ± 3	55 ± 8	85 ± 20

Summary statistics of mean sperm features of +/*t* and +/+ males over incubation time. Displayed are genotypic means and their standard errors. For any given incubation time, the means of the upper 50% (based on VCL) of motile sperm per individual male were averaged across 4–12 males, depending on incubation time. For % motile and sperm count, means from 8 scans per male were averaged across males.

VAP = Average path velocity; VSL = Straight-line velocity; VCL = Curvilinear velocity; ALH = Amplitude of lateral head displacement; BCF = Beat cross frequency; STR = Straightness; LI*n* = Linearity; % motile = Percentage of sperm that were motile, averaged for 8 scans per male; Count = Average number of sperm measured in each scan.

SUPPLEMENT C – THE COPULATORY PLUG DELAYS EJACULATION BY RIVAL MALES AND AFFECTS SPERM COMPETITION OUTCOME IN HOUSE MICE

SUPPLEMENTARY METHODS

Continuous variation in plug removal

In the main part of our study, we compared copulatory behaviour and paternity share between control and plug removal trials (see CHAPTER 5). However, in many plug removal trials we were unable to fully remove the plug. Thus, in an extension of our analyses of copulatory behaviour, we investigated variation in the size of the removed plug within plug removal trials. We visually estimated how much of the plug had been removed and weighed the removed piece of the plug to the nearest 0.1 mg. The proximal part of completely removed plugs typically showed a cup form corresponding to the form of the cervix, with a small central protrusion corresponding to the cervical orifice (picture inset in Figure S1). When plugs broke, typically the proximal part of the plug remained. Plugs appeared homogenous and there was no position at which plugs typically broke. We used the weight of the removed plug as a proxy for the size of the plug remaining in the female's vagina.

Validation of plug removal methodology – To validate this proxy, we ran additional monogamous mating trials to assess plug size variation in natural matings from this population. Mating trials were performed as in the plug removal experiment, but females were sacrificed after mating with a single male upon visual detection of a copulatory plug. The number of ejaculations was later counted from video observations. Plugs were dissected *post mortem* from the female genital tract. In analogy to the experimental plug removal trials, we visually assessed the extent of plug removal (here based on the amount of plug material remaining attached to the vaginal epithelium) and weighed the removed part to the nearest 0.1 mg.

SUPPLEMENTARY RESULTS AND DISCUSSION

Continuous variation in plug removal

There was substantial variation in the size of the removed plug piece. We compared the sizes of our experimentally removed plug pieces to plugs completely or partially removed *post mortem* after additional monogamous matings (Figure S1). We included information on the extent of plug removal (i.e., three categories: complete removal, majority removal, partial removal). In some of the trials, the male had ejaculated twice. Repeated ejaculation is associated with a decrease in plug size in house mice (CHAPTER 4), so we included plugs resulting from a second ejaculation as a fourth category. There was significant variation between the four categories (LMM: $F_{3,74} = 30.58$, $P < 0.001$), but experimentally removed plugs did not differ in size from plugs removed post mortem ($F_{1,38} = 0.01$, $P = 0.904$; Figure S1). For the remaining analyses, we thus used the size of the removed plug piece as a broad proxy for the size of the plug remaining inside the female (i.e., plug size). Because the plug piece remaining attached to the vaginal epithelium could not be quantified, the strength of the correlation between removed and remaining plug pieces could not be assessed more precisely.

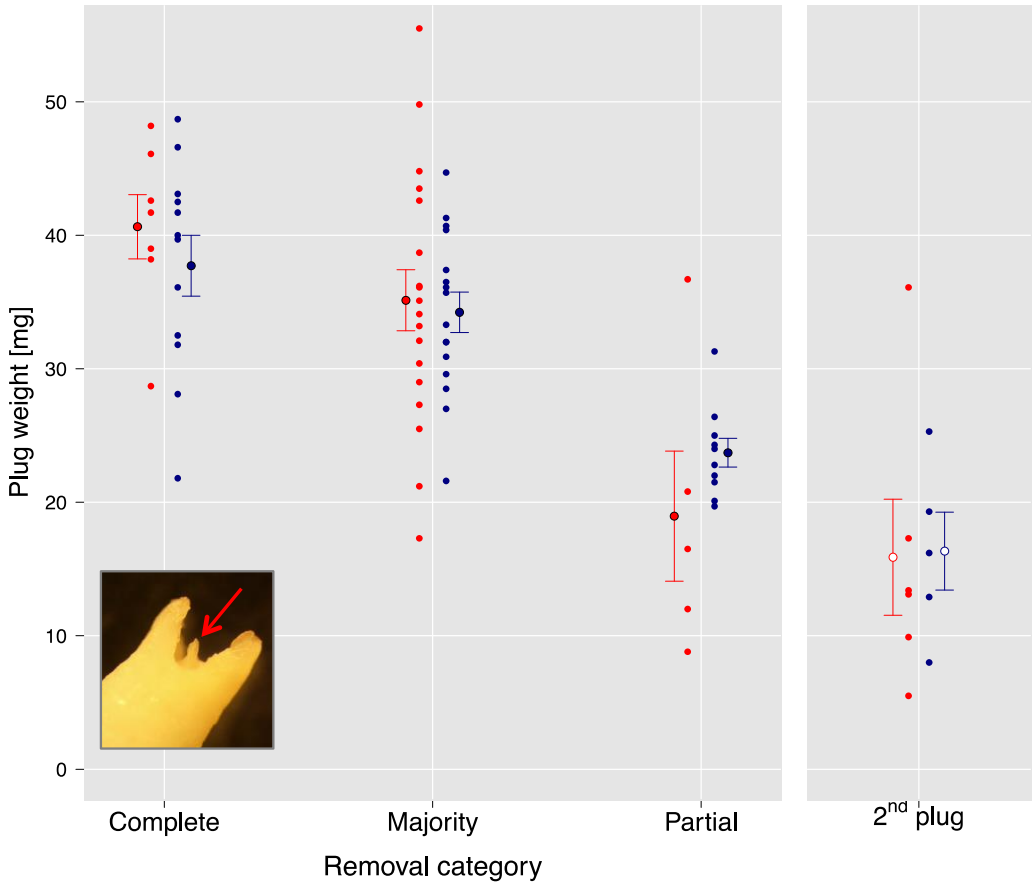

Figure S1

Experimental plug removal. Weights of the plug pieces removed in the main experiment (red) and in additional matings with post mortem plug removal (dark blue). Raw data are shown alongside with means and standard errors for different categories according to the estimated proportion of the plug removed. Plugs resulting from the males' second ejaculation (deposited after the male had removed his own 1st plug) are shown separately. Within categories, removal in the main experiment reflected post mortem removal. The picture inset shows the proximal tip of a completely removed copulatory plug. The red arrow highlights the central protrusion corresponding to the cervical orifice.

Copulatory behaviour

Using the subset of trials with copulatory plug removal, we ran LMMs on behavioural variables in analogy to step one—the contrast between control and plug removal trials (see Figure 5.1 and Table 5.1). We included the weight of the removed plug piece (our broad proxy for the size of the remaining plug) as a predictor variable, alongside standardised male and female body weight. Male identity nested within male family was included as a random effect. Neither mount latency (log transformed), the number and average duration (sqrt transformed) of copulatory bouts, nor *in copula* duration at ejaculation (sqrt transformed) were affected by our proxy of plug size (mount latency: $F_{1,27} = 0.001$, $P = 0.979$; N bouts: $F_{1,27} = 0.01$, $P = 0.935$; average duration: $F_{1,27} = 0.79$, $P = 0.383$; ejaculation duration: $F_{1,27} < 0.001$, $P = 0.985$). Removal of a larger piece of the

copulatory plug tended to be associated with a decrease in ejaculation latency (sqrt transformed) and ejaculation timing (log transformed), but these effects did not reach statistical significance (latency: b [95% c.i.] = -0.40 [-0.79, 0.001], $F_{1,27} = 3.30$, $P = 0.080$; timing: -0.011 [-0.022, 0.0003], $F_{1,27} = 3.15$, $P = 0.087$). Thus, the earlier ejaculation in the plug removal group may have been primarily caused by a reduction in plug size, rather than by additional unintended effects of the plug removal procedure on copulatory behaviour. Using the effect sizes obtained from trials with plug removal accurately predicted the latency and ejaculation timing in the control group (the latter is shown in Figure S2). However, an important caveat of the interpretation that larger plugs lead to a longer delay of ejaculation is that plug adherence rather than size could have lead to the observed effect. The remainder of plugs that were easier to experimentally remove may have also resisted removal by the second-to-mate male. Moreover, experimental plug removal could have depended on the size of the plug deposited. Recently, Mangels et al. (2015) showed that after monogamous matings, small plugs persisted in the female reproductive tract for longer than large plugs, suggesting that smaller plugs may be better at resisting proteolytic degredation by females. It is plausible that selection on males will not only act with respect to plug size but also with respect to plug adherence, over which there might be sexual conflict (Mangels et al., 2015). If larger plugs were easier to experimentally remove and the remainders of large plugs resisted rival male removal less than remainders of small plugs, the tendency for the size of the removed plug piece to correlate with rival male behaviour that we observed here (Figure S2) could have been driven by underlying size-associated differences in plug adherence.

The findings presented here are in line with recent findings from house mice that smaller plugs, caused by short male sexual rest, tended to correlate with males performing fewer copulatory bouts and ejaculating sooner (Chapter 4). However, due to our inability to determine the strength of the correlation between the sizes of the removed and remaining parts of the plug, this trend needs to be interpreted with caution. More dedicated experiments are needed to confirm the hypothesised effect of plug size on copulatory behaviour.

Paternity share

Using the subset of trials with copulatory plug removal, we ran a GLMM on P_1 to investigate whether plug removal may have affected paternity share through altering non-plug components of ejaculates (e.g., sperm numbers). Under such a scenario, one might expect a decrease of P_1 with a larger removed plug piece after the first male's mating even after controlling for the effect of ejaculation delay. Similarly, an increase of P_1 with a larger removed plug piece after the second male's mating could be expected.

After controlling for the effects of ejaculation delay, ejaculation numbers and male genotypes at the *t* locus (see main text), neither the size of the piece of the plug removed after the first nor that removed after the second male's mating had significant effects on P_1 ($P = 0.121$ and $P = 0.459$, respectively). In fact, the signs of the standardised effects were opposite to what could be expected if plug removal affected ejaculate components negatively (piece removed after first mating: b [95% CI] = 2.16 [-0.65, 4.97]; piece removed after second mating: -1.03 [-3.85, 1.79]). We interpret this as further evidence that the reduction in P_1 that was associated with plug removal was more likely caused by a decrease in the interval between the two males' ejaculations than through direct effects of plug removal on ejaculate components.

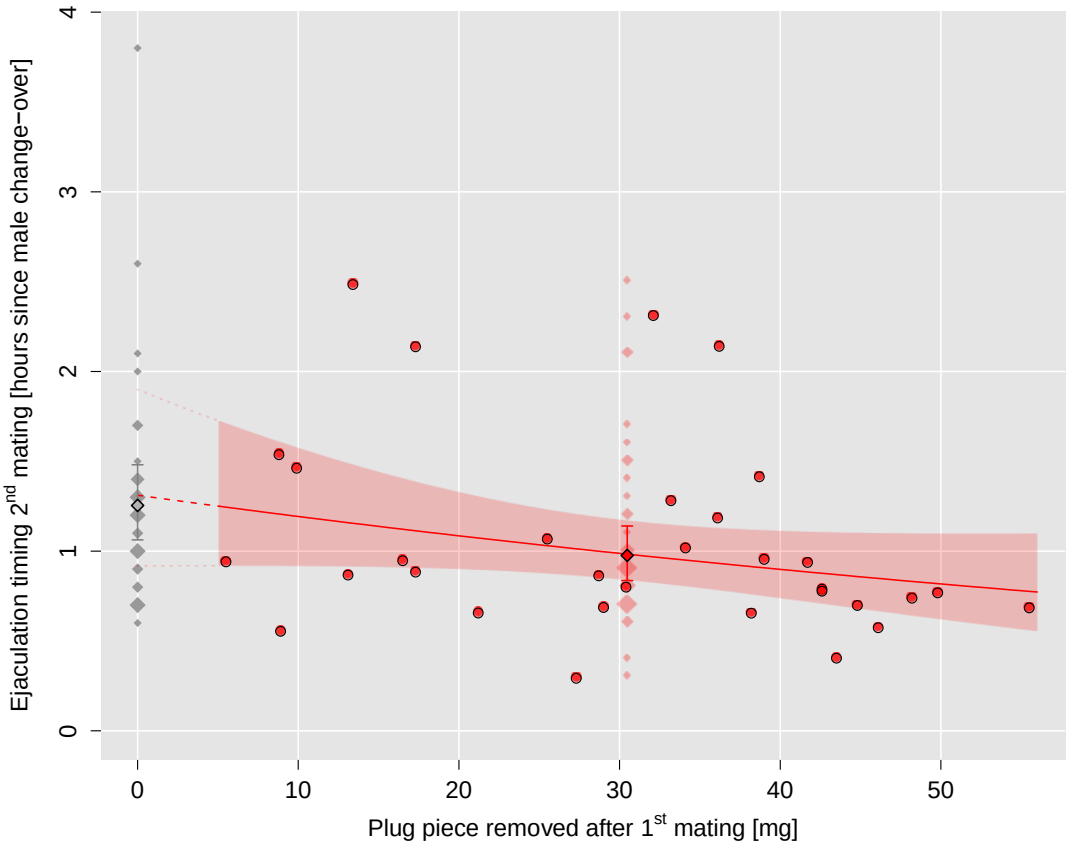

Figure S2

The timing of the second male's ejaculation as a function of the size of the piece of the first male's plug that had been removed. Red circles show continuous variation in the size of the plug piece removed. The red line and shaded area show the prediction and approximate confidence interval for the effect of the size of the removed plug piece on ejaculation timing from a model restricted to plug removal trials ($n = 32$). Although non-significant ($P = 0.087$), the extrapolated model estimate (dashed line) accurately predicted the ejaculation timing observed for the control treatment where plugs were not removed (grey diamonds). Diamonds illustrate the analysis presented in the main text, where treatment was fit as a categorical predictor (model prediction and approx. 95% CI highlighted, see Figure 5.1f and Table 5.1).

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